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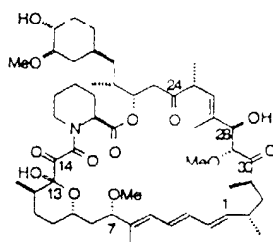
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| <p>(54) Title: REGULATION OF BIOLOGICAL EVENTS USING MULTIMERIC CHIMERIC PROTEINS<br/>(57) Abstract<br/>Materials and methods are disclosed for regulation of biological events such as target gene transcription and growth, proliferation or differentiation of engineered cells.</p>   |           |  |

## REGULATION OF BIOLOGICAL EVENTS USING MULTIMERIC CHIMERIC PROTEINS

## Background of the Invention

5 Rapamycin is a macrolide antibiotic produced by *Streptomyces hygroscopicus* which binds to a FK506-binding protein, FKBP, with high affinity to form a rapamycin:FKBP complex. Reported  $K_d$  values for that interaction are as low as 200 pM. The rapamycin:FKBP complex binds with high affinity to the large cellular protein, FRAP, to form a tripartite, [FKBP:rapamycin]:[FRAP], complex. In that complex rapamycin acts as a  
10 dimerizer or adapter to join FKBP to FRAP.



Rapamycin

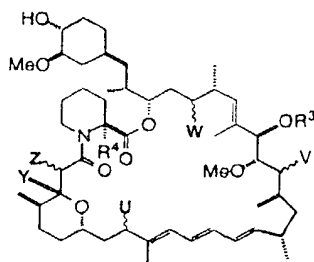
A number of naturally occurring FK506 binding proteins (FKBPs) are known. See e.g. Kay, 1996, *Biochem. J.* 314:361-385 (review). FKBP-derived domains have been incorporated in  
15 the design of chimeric proteins for use in biological switches in genetically engineered cells. Such switches rely upon ligand-mediated multimerization of the protein components to trigger a desired biological event. See e.g. Spencer et al, 1993, *Science* 262:1019-1024 and PCT/US94/01617. While the potent immunosuppressive activity of FK506 would limit its utility as a multimerizing agent, especially in animals, dimers of FK506 (and related  
20 compounds) can be made which lack such immunosuppressive activity. Such dimers have been shown to be effective for multimerizing chimeric proteins containing FKBP-derived ligand binding domains.

Rapamycin, like FK506, is also capable of multimerizing appropriately designed chimeric proteins. We have previously designed biological switches using rapamycin and various derivatives or analogs thereof ("rapalogs") as multimerizing agents (see  
25 WO96/41865). In the case of rapamycin itself, its significant biological activities, including potent immunosuppressive activity, rather severely limit its use in biological switches in certain applications, especially those in animals or animal cells which are sensitive to rapamycin. Improved rapalogs for such applications, especially rapalogs with reduced  
30 immunosuppressive activity, would be very desirable.

embodiments, however, the FKBP fusion protein is also an FRB fusion protein. In those  
embodiments, the chimeric protein comprises one or more FKBP domains as well as one or  
more FRB domains. In such cases, the first and second chimeric proteins may be the same  
protein, may be referred to as FKBP-FRB fusion proteins and contain at least one domain  
5 heterologous to the FKBP and/or FRB domains.

The chimeric proteins may be readily designed, based on incorporation of appropriately  
chosen heterologous domains, such that their multimerization triggers one or more of a wide  
variety of desired biological responses. The nature of the biological response triggered by  
rapalog-mediated complexation is determined by the choice of heterologous domains in the  
10 fusion proteins. The heterologous domains are therefore referred to as "action" or "effector"  
domains. The genetically engineered cells for use in practicing this invention will contain one  
or more recombinant nucleic acid constructs encoding the chimeric proteins, and in certain  
applications, will further contain one or more accessory nucleic acid constructs, such as one or  
more target gene constructs. Illustrative biological responses, applications of the system and  
15 types of accessory nucleic acid constructs are discussed in detail below.

A system involving related materials and methods is disclosed in WO 96/41865  
(Clackson et al) and is expected to be useful in a variety of applications including, among  
others, research uses and therapeutic applications. That system involves the use of a  
multimerizing agent comprising rapamycin or a rapalog of the generic formula:

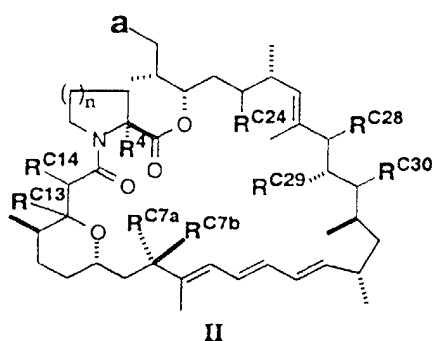


wherein U is -H, -OR<sup>1</sup>, -SR<sup>1</sup>, -OC(O)R<sup>1</sup>, -OC(O)NHR<sup>1</sup>, -NHR<sup>1</sup>, -NHC(O)R<sup>1</sup>, -NHSO<sub>2</sub>-R<sup>1</sup> or  
-R<sup>2</sup>; R<sup>2</sup> is a substituted aryl or allyl or alkylaryl (e.g. benzyl or substituted benzyl); V is-  
OR<sup>3</sup> or (=O); W is =O, =NR<sup>4</sup>, =NOR<sup>4</sup>, =NNHR<sup>4</sup>, -NHOR<sup>4</sup>, -NHNHR<sup>4</sup>, -OR<sup>4</sup>, -OC(O)R<sup>4</sup>, -  
OC(O)NR<sup>4</sup> or -H; Y is -OR<sup>5</sup>, -OC(O)R<sup>5</sup> or -OC(O)NHR<sup>5</sup>; Z is =O, -OR<sup>6</sup>, -NR<sup>6</sup>, -H, -  
25 NC(O)R<sup>6</sup>, -OC(O)R<sup>6</sup> or -OC(O)NR<sup>6</sup>; R<sup>3</sup> is H, -R<sup>7</sup>, -C(O)R<sup>7</sup>, -C(O)NHR<sup>7</sup> or C-28 / C-30 cyclic  
carbonate; and R<sup>4</sup> is H or alkyl; where R<sup>1</sup>, R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup> and R<sup>7</sup> are independently selected from  
H, alkyl, alkylaryl or aryl, as those terms are defined in WO 96/41865. A number of  
rapalogs are specifically disclosed in that document.

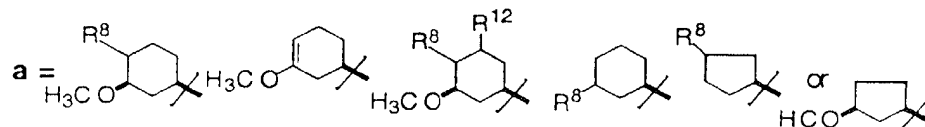
assay is predictive of a decrease in immunosuppressive activity in humans, relative to rapamycin. Such in vitro assays may be used to evaluate the rapalog's relative immunosuppressive activity.

- 5 A variety of illustrative examples of such rapalogs are disclosed herein. This class of improved rapalogs includes, among others, those which bind to human FKBP12, or inhibit its rotamase activity, within an order of magnitude of results obtained with rapamycin in any conventional FKBP binding or rotamase assay.

Other classes of improved rapalogs for use in this invention are defined with reference to the structure shown in Formula II:



wherein



and,

- 15 one of  $R^{C7a}$  and  $R^{C7b}$  is H and the other is -H, halo,  $-R^2$ ,  $-OR^1$ ,  $-SR^1$ ,  $-OC(O)R^1$  or  $-OC(O)NHR^1$ ,  $-NHR^1$ ,  $-NR^1R^2$ ,  $-NHC(O)R^1$ , or  $-NH-SO_2-R^1$  where  $R^2$  = aliphatic, heteroaliphatic, aryl, heteroaryl or alkylaryl (e.g. benzyl or substituted benzyl);

$R^{C30}$  is halo,  $-OR^3$  or  $(=O)$ ;

$R^{C24}$  is  $-O$ ,  $=NR^4$ ,  $=NOR^4$ ,  $=NNHR^4$ ,  $-NHOR^4$ ,  $-NHNHR^4$ ,  $-OR^4$ ,  $-OC(O)R^4$  or

- 20  $-OC(O)NR^4$ , halo or  $-H$ ;

$R^{C13}$  and  $R^{C28}$  are independently H, halo,  $-OR^3$ ,  $-OR^5$ ,  $-OC(O)R^5$ ,  $-OC(O)NHR^5$ ,  $-SR^5$ ,  $-SC(O)R^5$ ,  $-SC(O)NHR^5$ ,  $-NR^5R^{5'}$  or  $-N(R^5)(CO)R^{5'}$ ;

$R^{C14}$  is  $=O$ ,  $-OR^6$ ,  $-NR^6$ ,  $-H$ ,  $-NC(O)R^6$ ,  $-OC(O)R^6$  or  $-OC(O)NR^6$ ;

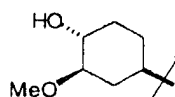
Another class of improved rapalogs for use in this invention which are of particular interest are rapalogs of Formula II wherein  $RC^{7a}$  and  $RC^{7b}$  are moieties other than a substituted or unsubstituted allyl group or a methoxy moiety. This class includes rapalogs in which one of  $RC^{7a}$  and  $RC^{7b}$  is H and the other is phenyl, di- or tri-substituted phenyl or a mono- or di-substituted heterocyclic moiety. Illustrative examples include among others, o,p-dialkoxyphenyl substituents (e.g., o,p-dimethoxyphenyl, o-methoxy-p-ethoxyphenyl, o-ethoxy-p-methoxyphenyl, o,p-diethoxyphenyl, o,p-di (n- or iso-)propoxyphenyl, etc.), trialkoxyphenyl substituents, monosubstituted heterocycles such as methylthiophene, etc. One subset of such compounds differs in structure from rapamycin only at  $RC^{24}$  and  $RC^{30}$ .

- 5 Another subset of such compounds differs in structure from rapamycin at one or more additional position, as set forth above in connection with Formula II or in connection with any of the other classes of improved rapalogs noted herein.

Another class of improved rapalogs for use in this invention which are of particular interest are rapalogs of Formula II wherein n is 1. This class of rapalogs includes rapalogs comprising a prolyl ring system in place of a pipicolate ring system. One subset of such compounds differs in structure from rapamycin only with respect to the pipicolate ring system. Another subset of such compounds differs in structure from rapamycin with respect to one or more additional structural features (e.g. one or both substituents at C7, for instance), as set forth above in connection with Formula II or in connection with any of the other classes of improved rapalogs noted herein.

- 15 Another subset of such compounds differs in structure from rapamycin only with respect to the pipicolate ring system. Another subset of such compounds differs in structure from rapamycin with respect to one or more additional structural features (e.g. one or both substituents at C7, for instance), as set forth above in connection with Formula II or in connection with any of the other classes of improved rapalogs noted herein.

Another class of improved rapalogs for use in this invention which are of particular interest are rapalogs of Formula II wherein moiety "a" is other than



One subset of such compounds differs in structure from rapamycin only with respect to the ring system, "a". Another subset of such compounds differs in structure from rapamycin with respect to one or more additional structural features (e.g. one or both substituents at C7, for instance), as set forth above in connection with Formula II or in connection with any of the other classes of improved rapalogs noted herein. This class of rapalogs include the class of 43-epi-rapalogs in which the hydroxyl moiety at position 43 has the opposite stereochemical orientation with that shown immediately above, is a mixture of stereoisomers of the 43-hydroxyl group or contains derivatives of any of the foregoing, including ethers, esters, carbamates, halides and other derivatives of any of the foregoing position 43 rapalogs. This class further includes rapalogs in which the cyclohexyl ring is

- 25 Another subset of such compounds differs in structure from rapamycin only with respect to the ring system, "a". Another subset of such compounds differs in structure from rapamycin with respect to one or more additional structural features (e.g. one or both substituents at C7, for instance), as set forth above in connection with Formula II or in connection with any of the other classes of improved rapalogs noted herein. This class of rapalogs include the class of 43-epi-rapalogs in which the hydroxyl moiety at position 43 has the opposite stereochemical orientation with that shown immediately above, is a mixture of stereoisomers of the 43-hydroxyl group or contains derivatives of any of the foregoing, including ethers, esters, carbamates, halides and other derivatives of any of the foregoing position 43 rapalogs. This class further includes rapalogs in which the cyclohexyl ring is

In certain embodiments the chimeric protein(s) contain at least one modification in peptide sequence, preferably up to three modifications, relative to naturally occurring sequences, in both one or more FKBP domains and one or more FRB domains.

As mentioned previously, in the various embodiments of this invention, the chimeric protein(s) contain one or more "action" or "effector" domains which are heterologous with respect to the FKBP and/or FRB domains. Effector domains may be selected from a wide variety of protein domains including DNA binding domains, transcription activation domains, cellular localization domains and signaling domains (i.e., domains which are capable upon clustering or multimerization, of triggering cell growth, proliferation, differentiation, apoptosis, gene transcription, etc.). A variety of illustrative effector domains which may be used in practicing this invention are disclosed in the various scientific and patent documents cited herein.

For example, in certain embodiments, one fusion protein contains at least one DNA binding domain (e.g., a GAL4 or ZFHD1 DNA-binding domain) and another fusion protein contains at least one transcription activation domain (e.g., a VP16 or p65 transcription activation domain). Ligand-mediated association of the fusion proteins represents the formation of a transcription factor complex and leads to initiation of transcription of a target gene linked to a DNA sequence recognized by (i.e., capable of binding with) the DNA-binding domain on one of the fusion proteins.

In other embodiments, one fusion protein contains at least one domain capable of directing the fusion protein to a particular cellular location such as the cell membrane, nucleus, ER or other organelle or cellular component. Localization domains which target the cell membrane, for example, include domains such as a myristoylation site or a transmembrane region of a receptor protein or other membrane-spanning protein. Another fusion protein can contain a signaling domain capable, upon membrane localization and/or clustering, of activating a cellular signal transduction pathway. Examples of signaling domains include an intracellular domain of a growth factor or cytokine receptor, an apoptosis triggering domain such as the intracellular domain of FAS or TNF-R1, and domains derived from other intracellular signaling proteins such as SOS, Raf, lck, ZAP-70, etc. A number of signaling proteins are disclosed in PCT/US94/01617 (see e.g. pages 23 - 26). In still other embodiments, each of the fusion proteins contains at least one FRB domain and at least one FKBP domain, as well as one or more heterologous domains. Such fusion proteins are capable of homodimerization and triggering signaling in the presence of the rapalog. In general, domains containing peptide sequence endogenous to the host cell are preferred in

preferably eukaryotic cells, more preferably animal cells, and most preferably mammalian cells. Primate cells, especially human cells, are of particular interest. Administration of the improved rapalog to a human or non-human animal may be effected using any pharmaceutically acceptable formulation and route of administration. Oral administration  
5 of a pharmaceutically acceptable composition containing the improved rapalog together with one or more pharmaceutically acceptable carriers, buffers or other excipients is currently of greatest interest.

A specific object of this invention is a method, as otherwise described above, for inducing transcription of a target gene in a rapalog-dependent manner. The cells typically contain, in  
10 addition to recombinant DNAs encoding the two fusion proteins, a target gene construct which comprises a target gene operably linked to a DNA sequence which is responsive to the presence of a complex of the fusion proteins with rapamycin or a rapalog. The target gene construct may be recombinant, and the target gene and/or a regulatory nucleic acid sequence linked thereto may be heterologous with respect to the host cell. In certain embodiments the  
15 cells are responsive to contact with an improved rapalog which binds to the FKBP fusion protein and participates in a complex with a FRB fusion protein with a detectable preference over binding to endogenous FKBP and/or FRB-containing proteins of the host cell.

Another specific object of this invention is a method, as otherwise described above, for inducing cell death in a rapalog-dependent manner. In such cells, at least one of the  
20 heterologous domains on at least one fusion protein, and usually two fusion proteins, is a domain such as the intracellular domain of FAS or TNF-R1, which, upon clustering, triggers apoptosis of the cell.

Another specific object of this invention is a method, as otherwise described above, for inducing cell growth, differentiation or proliferation in a rapalog-dependent manner. In  
25 such cells, at least one of the heterologous domains of at least one of the fusion proteins is a signaling domain such as, for example, the intracellular domain of a receptor for a hormone which mediates cell growth, differentiation or proliferation, or a downstream mediator of such a receptor. Cell growth, differentiation and/or proliferation follows clustering of such signalling domains. Such clustering occurs in nature following hormone binding, and in  
30 engineered cells of this invention following contact with an improved rapalog.

Cells of human origin are preferred for human gene therapy applications, although cell types of various origins (human or other species) may be used, and may, if desired, be encapsulated within a biocompatible material for use in human subjects.

Also disclosed are kits for use in the genetic engineering of cells or human or non-human animals as described herein. One such kit contains one or more recombinant nucleic acid constructs encoding fusion proteins of this invention. The recombinant nucleic acid constructs will generally be in the form of eukaryotic expression vectors suitable for introduction into animal cells and capable of directing the expression of the fusion proteins therein. Such vectors may be viral vectors as described elsewhere herein. The kit may also contain a sample of an improved rapalog of this invention capable of forming a complex with the encoded fusion proteins. The kit may further contain a multimerization antagonist such as FK506 or some other compound capable of binding to one of the fusion proteins but incapable of forming a complex with both. In certain embodiments, the recombinant nucleic acid constructs encoding the fusion proteins will contain a cloning site in place of DNA encoding one or more of the heterologous domains, thus permitting the practitioner to introduce DNA encoding a heterologous domain of choice. In some embodiments the kit may also contain a target gene construct containing a target gene or cloning site linked to a DNA sequence responsive to the presence of the complexed fusion proteins, as described in more detail elsewhere. The kit may contain a package insert identifying the enclosed nucleic acid construct(s), and/or instructions for introducing the construct(s) into host cells or organisms.

#### Brief Description of the Figures

Figure 1 demonstrates the ability of 13-F-rapalogs (compounds 79 and 108, synthesized as described in Examples 6.1 and 6.21, respectively) to stimulate expression of a DNA sequence encoding secreted alkaline phosphatase ("SEAP") in HT1080 cells engineered as described in Example 7.

Figure 2 depicts the results of transcription assays using rapalogs 42, 53, 69 and 96, synthesized as described herein, as dimerizer. Rapalog s were tested in cells expressing wild-type FRB (Figs. 2A and 2C) as well as in cells expressing a mutant FRB in which Thr 2098 was replaced by Leu (Figs 2B and 2D) or by Phe (Fig 2E).

#### Detailed Description of the Invention

##### Definitions

The definitions and orienting information below will be helpful for a full understanding of this document.



related references cited herein). The peptide sequence of such an FRB domain comprises (a) a naturally occurring peptide sequence spanning at least the indicated 89-amino acid region of the proteins noted above or corresponding regions of homologous proteins; (b) a variant of a naturally occurring FRB sequence in which up to about ten (preferably 1-5, more preferably 1-3, and in some embodiments just one) amino acids of the naturally-occurring peptide sequence have been deleted, inserted, or replaced with substitute amino acids; or (c) a peptide sequence encoded by a DNA sequence capable of selectively hybridizing to a DNA molecule encoding a naturally occurring FRB domain or by a DNA sequence which would be capable, but for the degeneracy of the genetic code, of selectively hybridizing to a DNA molecule encoding a naturally occurring FRB domain.

FKBPs (FK506 binding proteins) are the cytosolic receptors for macrolides such as FK506, FK520 and rapamycin and are highly conserved across species lines. For the purpose of this disclosure, FKBP are proteins or protein domains which are capable of binding to rapamycin or to an improved rapalog of this invention and further forming a tripartite complex with an FRB-containing protein. An FKBP domain may also be referred to as a "rapamycin binding domain". Information concerning the nucleotide sequences, cloning, and other aspects of various FKBP species is already known in the art, permitting the synthesis or cloning of DNA encoding the desired FKBP peptide sequence, e.g., using well known methods and PCR primers based on published sequences. See e.g. Staendert et al, 1990, Nature 346, 671-674 (human FKBP12); Kay, 1996, Biochem. J. 314, 361-385 (review). Homologous FKBP proteins in other mammalian species, in yeast, and in other organisms are also known in the art and may be used in the fusion proteins disclosed herein. See e.g. Kay, 1996, Biochem. J. 314, 361-385 (review). The size of FKBP domains for use in this invention varies, depending on which FKBP protein is employed. An FKBP domain of a fusion protein of this invention will be capable of binding to rapamycin or an improved rapalog of this invention and participating in a tripartite complex with an FRB-containing protein (as may be determined by any means, direct or indirect, for detecting such binding). The peptide sequence of an FKBP domain of an FKBP fusion protein of this invention comprises (a) a naturally occurring FKBP peptide sequence, preferably derived from the human FKBP12 protein (exemplified below) or a peptide sequence derived from another human FKBP, from a murine or other mammalian FKBP, or from some other animal, yeast or fungal FKBP; (b) a variant of a naturally occurring FKBP sequence in which up to about ten (preferably 1-5, more preferably 1-3, and in some embodiments just one) amino acids of the naturally-occurring peptide sequence have been deleted, inserted, or replaced with substitute amino acids; or (c) a peptide sequence encoded by a DNA sequence capable of selectively hybridizing to a DNA molecule encoding a naturally occurring FKBP or by a DNA sequence

promoter. Such transcription regulatory components can be present upstream of a coding region, or in certain cases (e.g. enhancers), in other locations as well, such as in introns, exons, coding regions, and 3' flanking sequences.

"Dimerization", "oligomerization" and "multimerization" are used interchangeably  
5 herein and refer to the association or clustering of two or more protein molecules, mediated by the binding of a drug to at least one of the proteins. In preferred embodiments, the multimerization is mediated by the binding of two or more such protein molecules to a common divalent or multivalent drug. The formation of a complex comprising two or more protein molecules, each of which containing one or more FKBP domains, together with one or  
10 more molecules of an FKBP ligand which is at least divalent (e.g. FK1012 or AP1510) is an example of such association or clustering. In cases where at least one of the proteins contains more than one drug binding domain, e.g., where at least one of the proteins contains three FKBP domains, the presence of a divalent drug leads to the clustering of more than two protein molecules. Embodiments in which the drug is more than divalent (e.g. trivalent) in  
15 its ability to bind to proteins bearing drug binding domains also can result in clustering of more than two protein molecules. The formation of a tripartite complex comprising a protein containing at least one FRB domain, a protein containing at least one FKBP domain and a molecule of rapamycin is another example of such protein clustering. In certain embodiments of this invention, fusion proteins contain multiple FRB and/or FKBP domains. Complexes of  
20 such proteins may contain more than one molecule of rapamycin or a derivative thereof or other dimerizing agent and more than one copy of one or more of the constituent proteins. Again, such multimeric complexes are still referred to herein as tripartite complexes to indicate the presence of the three types of constituent molecules, even if one or more are represented by multiple copies. The formation of complexes containing at least one divalent  
25 drug and at least two protein molecules, each of which contains at least one drug binding domain, may be referred to as "oligomerization" or "multimerization", or simply as "dimerization", "clustering" or "association".

"Dimerizer" denotes an improved rapalog of this invention which brings together two or more proteins in a multimeric complex.

30 "Activate" as applied herein to the expression or transcription of a gene denotes a directly or indirectly observable increase in the production of a gene product.

"Genetically engineered cells" denotes cells which have been modified ("transduced") by the introduction of recombinant or heterologous nucleic acids (e.g. one or more DNA

PCT/US94/08008 and Spencer et al, supra. The design and use of such chimeric proteins for ligand-mediated gene-knock out and for ligand-mediated blockade of gene expression or inhibition of gene product function are disclosed in PCT/US95/10591. Novel DNA binding domains and DNA sequences to which they bind which are useful in embodiments involving regulated transcription of a target gene are disclosed, e.g., in Pomeranz et al, 1995, Science 267:93-96. Those references provide substantial information, guidance and examples relating to the design, construction and use of DNA constructs encoding analogous chimeras, target gene constructs, and other aspects which may also be useful to the practitioner of the subject invention.

By appropriate choice of chimeric proteins, this invention permits one to activate the transcription of a desired gene; actuate cell growth, proliferation, differentiation or apoptosis; or trigger other biological events in engineered cells in a rapalog-dependent manner analogous to the systems described in the patent documents and other references cited above. The engineered cells, preferably animal cells, may be growing or maintained in culture or may be present within whole organisms, as in the case of human gene therapy, transgenic animals, and other such applications. The rapalog is administered to the cell culture or to the organism containing the engineered cells, as the case may be, in an amount effective to multimerize the FKBP fusion proteins and FRB fusion proteins (as may be observed indirectly by monitoring target gene transcription, apoptosis or other biological process so triggered). In the case of administration to whole organisms, the rapalog may be administered in a composition containing the rapalog and one or more acceptable veterinary or pharmaceutical diluents and/or excipients.

A compound which binds to one of the chimeric proteins but does not form tripartite complexes with both chimeric proteins may be used as a multimerization antagonist. As such it may be administered to the engineered cells, or to organisms containing them (preferably in a composition as described above in the case of administration to whole animals), in an amount effective for blocking or reversing the effect of the rapalog, i.e. for preventing, inhibiting or disrupting multimerization of the chimeras. For instance, FK506, FK520 or any of the many synthetic FKBP ligands which do not form tripartite complexes with FKBP and FRAP may be used as an antagonist.

One important aspect of this invention provides materials and methods for rapalog-dependent, direct activation of transcription of a desired gene. In one such embodiment, a set of two or more different chimeric proteins, and corresponding DNA constructs capable of directing their expression, is provided. One such chimeric protein contains as its action

Embodiments involving regulatable actuation of apoptosis provide engineered cells susceptible to rapalog-inducible cell death. Such engineered cells can be eliminated from a cell culture or host organism after they have served their intended purposed (e.g. production of a desired protein or other product), if they have or develop unwanted properties, or if  
5 they are no longer useful, safe or desired. Elimination is effected by adding the rapalog to the medium or administering it to the host organism. In such cases, the action domains of the chimeras are protein domains such as the intracellular domains of FAS or TNF-R1, downstream components of their signaling pathways or other protein domains which upon oligomerization trigger apoptosis.

10 This invention thus provides materials and methods for achieving a biological effect in cells in response to the addition of a rapalog of this invention. The method involves providing cells engineered as described herein and exposing the cells to the rapalog.

For example, this invention provides a method for activating transcription of a target gene in cells. The method involves providing cells containing (a) DNA constructs encoding a  
15 set of chimeric proteins of this invention capable upon rapalog-mediated multimerization of initiating transcription of a target gene and (b) a target gene linked to an associated cognate DNA sequence responsive to the multimerization event (e.g. a DNA sequence recognized, i.e., capable of binding with, a DNA-binding domain of a foregoing chimeric protein. The method involves exposing the cells to a rapalog capable of binding to the chimeric proteins  
20 in an amount effective to result in expression of the target gene. In cases in which the cells are growing in culture, exposing the cells to the rapalog may be effected by adding the rapalog to the culture medium. In cases in which the cells are present within a host organism, exposing them to the rapalog is effected by administering the rapalog to the host organism. For instance, in cases in which the host organism is a human or non-human, the  
25 rapalog may be administered to the host organism by oral, bucal, sublingual, transdermal, subcutaneous, intramuscular, intravenous, intra-joint or inhalation administration in an appropriate vehicle therefor. Again, depending on the design of the constructs for the chimeric proteins and of any accessory constructs, the rapalog-mediated biological event may be activation of a cellular function such as signal transduction leading to cell growth,  
30 cell proliferation, gene transcription, or apoptosis; deletion of a gene of interest, blockade of expression of a gene of interest, or inhibition of function of a gene product of interest; direct transcription of a gene of interest; etc.

This invention further encompasses a pharmaceutical composition comprising a rapalog of this invention in admixture with a pharmaceutically acceptable carrier and optionally

Also provided are kits for producing cells responsive to a rapalog of this invention. One such kit contains one or more nucleic acid constructs encoding and capable of directing the expression of chimeras which, upon rapalog-mediated oligomerization, trigger the desired biological response. The kit may contain a quantity of a rapalog capable of multimerizing the chimeric protein molecules encoded by the construct(s) of the kit, and may contain in addition a quantity of a multimerization antagonist. The kit may further contain a nucleic acid construct encoding a target gene (or cloning site) linked to a cognate DNA sequence which is recognized by the dimerized chimeric proteins permitting transcription of a gene linked to that cognate DNA sequence in the presence of multimerized chimeric protein molecules. The constructs may be associated with one or more selection markers for convenient selection of transfectants, as well as other conventional vector elements useful for replication in prokaryotes, for expression in eukaryotes, and the like. The selection markers may be the same or different for each different construct, permitting the selection of cells which contain each such construct(s).

The accessory construct for introducing into cells a target gene in association with a cognate DNA sequence may contain a cloning site in place of a target gene. A kit containing such a construct permits the engineering of cells for regulatable expression of a gene to be provided by the practitioner.

Other kits of this invention may contain one or two (or more) nucleic acid constructs for chimeric proteins in which one or more contain a cloning site in place of the transcriptional activator or DNA binding protein, permitting the user to insert whichever such domain s/he wishes. Such a kit may optionally include other elements as described above, e.g. a nucleic acid construct for a target gene with or without a cognate DNA sequence for a pre-selected DNA binding domain.

Any of the kits may also contain positive control cells which were stably transformed with constructs of this invention such that they express a reporter gene (for CAT, beta-galactosidase or any conveniently detectable gene product) in response to exposure of the cells to the rapalog. Reagents for detecting and/or quantifying the expression of the reporter gene may also be provided.

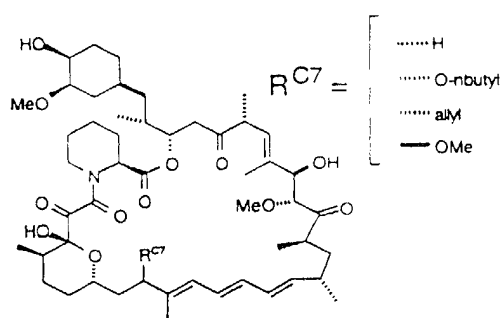
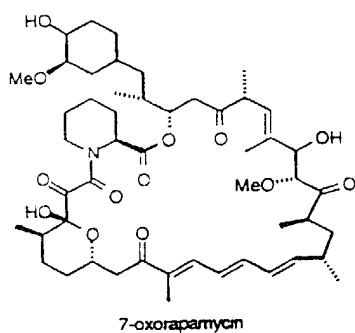
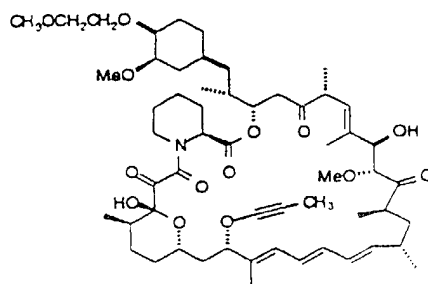
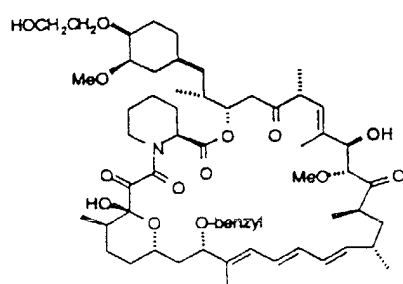
For further information and guidance on the design, construction and use of such systems or components thereof which may be adapted for use in practising the subject invention, reference to the following publications is suggested: Spencer et al, 1993, *supra*; Rivera et al, 1996, *supra*; Spencer et al, 1996, *Current Biology* 6, 839-847; Luo et al, 1996, *Nature*, 383, 181-185; Ho et al, 1996, *Nature* 382, 822-826; Belshaw et al, 1996, *Proc. Natl. Acad. Sci. USA*

C14, C24 and/or C30; replacement of the 6-membered pipecolate ring with a 5-membered prolyl ring; and elimination, derivatization or replacement of one or more substituents of the cyclohexyl ring or replacement of the cyclohexyl ring with a substituted or unsubstituted cyclopentyl ring. Rapalogs, as that term is used herein, do not include rapamycin itself, and preferably do not contain an oxygen bridge between C1 and C30. Illustrative examples of rapalogs are disclosed in the documents listed in Table I. Examples of rapalogs modified at C7 are shown in Table II.

Table I

|           |            |           |           |           |
|-----------|------------|-----------|-----------|-----------|
| WO9710502 | WO9418207  | WO9304680 | US5527907 | US5225403 |
| WO9641807 | WO9410843  | WO9214737 | US5484799 | US5221625 |
| WO9635423 | WO9409010  | WO9205179 | US5457194 | US5210030 |
| WO9603430 | WO94/04540 | US5604234 | US5457182 | US5208241 |
| WO9600282 | WO9402485  | US5597715 | US5362735 | US5200411 |
| WO9516691 | WO9402137  | US5583139 | US5324644 | US5198421 |
| WO9515328 | WO9402136  | US5563172 | US5318895 | US5147877 |
| WO9507468 | WO9325533  | US5561228 | US5310903 | US5140018 |
| WO9504738 | WO9318043  | US5561137 | US5310901 | US5116756 |
| WO9504060 | WO9313663  | US5541193 | US5258389 | US5109112 |
| WO9425022 | WO9311130  | US5541189 | US5252732 | US5093338 |
| WO9421644 | WO9310122  | US5534632 | US5247076 | US5091389 |

Table II (cont'd):



*See e.g.,*

Luengo *et al*, Chemistry & Biology, 1995, 2 (7):471-481; JOC, 1995, 59(22):6512-13

WO 94/02136 (SmithKline Beecham)

WO 95/16691 (Sandoz)

US 5583139 (Abbott)

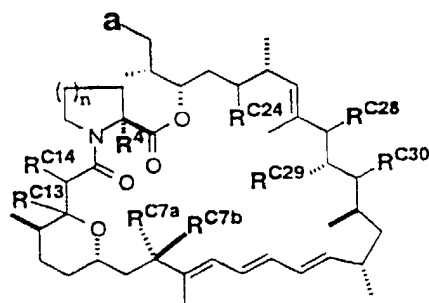
Grinfeld *et al*, 1994, Tett Letters 35(37):6835-6838

WO 96/41865 (ARIAD)

Table III (cont'd)

|   |  |
|---|--|
|   |  |
|   |  |
| <p>TBDMS- = <math>t\text{BuMe}_2\text{Si-}</math></p> |  |

Rapalogs of particular interest for the practice of various aspects of this invention include compounds of formula II:



II



practising the subject invention. Thus the improved rapalogs of this invention are rapalogs other than those depicted in Table III.

In rapamycin,  $R^{C7a}$  is -OMe;  $R^{C7b}$  is H;  $R^{C14}$ ,  $R^{C24}$  and  $R^{C30}$  are each (=O);  $R^{C13}$  and  $R^{C28}$  are each -OH;  $R^{C29}$  is OMe; and  $R^3$  and  $R^4$  are each H, all with the stereoisomerism as shown on page 1. Rapalogs useful in practicing this invention may contain substituents in any of the possible stereoisomeric orientations, and may comprise one stereoisomer substantially free of other stereoisomers (>90%, and preferably >95%, free from other stereoisomers on a molar basis) or may comprise a mixture of stereoisomers.

Also included are pharmaceutically acceptable derivatives of the foregoing compounds, where the phrase "pharmaceutically acceptable derivative" denotes any pharmaceutically acceptable salt, ester, or salt of such ester, of such compound, or any other adduct or derivative which, upon administration to a patient, is capable of providing (directly or indirectly) a rapalog as described herein, or a metabolite or residue thereof. Pharmaceutically acceptable derivatives thus include among others pro-drugs of the rapalogs. A pro-drug is a derivative of a compound, usually with significantly reduced pharmacological activity, which contains an additional moiety which is susceptible to removal *in vivo* yielding the parent molecule as the pharmacologically active species. An example of a pro-drug is an ester which is cleaved *in vivo* to yield a compound of interest. Various pro-drugs of rapamycin and of other compounds, and materials and methods for derivatizing the parent compounds to create the pro-drugs, are known and may be adapted to the present invention.

The term "aliphatic" as used herein includes both saturated and unsaturated, straight chain (*i.e.*, unbranched), branched, cyclic, or polycyclic aliphatic hydrocarbons, which are optionally substituted with one or more functional groups. Unless otherwise specified, alkyl, other aliphatic, alkoxy and acyl groups preferably contain 1-8, and in many cases 1-6, contiguous aliphatic carbon atoms. Illustrative aliphatic groups thus include, for example, methyl, ethyl, n-propyl, isopropyl, cyclopropyl, -CH<sub>2</sub>-cyclopropyl, allyl, n-butyl, sec-butyl, isobutyl, tert-butyl, cyclobutyl, -CH<sub>2</sub>-cyclobutyl, n-pentyl, sec-pentyl, isopentyl, tert-pentyl, cyclopentyl, -CH<sub>2</sub>-cyclopentyl, n-hexyl, sec-hexyl, cyclohexyl, -CH<sub>2</sub>-cyclohexyl moieties and the like, which again, may bear one or more substituents.

Examples of substituents include: -OH, -OR<sup>2'</sup>, -SH, -SR<sup>2'</sup>, -CHO, =O, -COOH (or ester, carbamate, urea, oxime or carbonate thereof), -NH<sub>2</sub> (or substituted amine, amide, urea, carbamate or guanidino derivative thereof), halo, trihaloalkyl, cyano, -SO<sub>2</sub>-CF<sub>3</sub>, -OSO<sub>2</sub>F, -OS(O)<sub>2</sub>R<sup>11</sup>, -SO<sub>2</sub>-NHR<sup>11</sup>, -NHSO<sub>2</sub>-R<sup>11</sup>, sulfate, sulfonate, aryl and heteroaryl moieties. Aryl

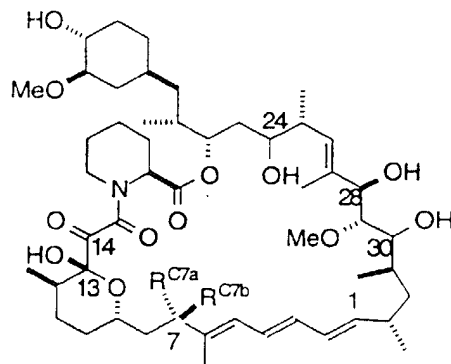
tetrahydrofuranyl, tetrahydropyranyl, aziridine, azetidine, pyrrolidine, piperidine, morpholine, piperazine and the like.

The terms "aryl" and "heteroaryl" as used herein refer to stable mono- or polycyclic, heterocyclic, polycyclic, and polyheterocyclic unsaturated moieties having 3 - 14 carbon atoms which may be substituted or unsubstituted. Substituents include any of the previously mentioned substituents. Non-limiting examples of useful aryl ring groups include phenyl, halophenyl, alkoxyphenyl, dialkoxyphenyl, trialkoxyphenyl, alkylenedioxyphenyl, naphthyl, phenanthryl, anthryl, phenanthro and the like. Examples of typical heteroaryl rings include 5-membered monocyclic ring groups such as thienyl, pyrrolyl, imidazolyl, pyrazolyl, furyl, isothiazolyl, furazanyl, isoxazolyl, thiazolyl and the like; 6-membered monocyclic groups such as pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, triazinyl and the like; and polycyclic heterocyclic ring groups such as benzo[b]thienyl, naphtho[2,3-b]thienyl, thianthrenyl, isobenzofuranyl, chromenyl, xanthenyl, phenoxathienyl, indoliziny, isoindolyl, indolyl, indazolyl, purinyl, isoquinolyl, quinolyl, phthalazinyl, naphthyridinyl, quinoxalinyl, quinazolinyl, benzothiazole, benzimidazole, tetrahydroquinoline cinnolinyl, pteridinyl, carbazolyl, beta-carbolinyl, phenanthridinyl, acridinyl, perimidinyl, phenanthrolinyl, phenazinyl, isothiazolyl, phenothiazinyl, phenoxazinyl, and the like (see *e.g.* Katritzky, Handbook of Heterocyclic Chemistry). The aryl or heteroaryl moieties may be substituted with one to five members selected from the group consisting of hydroxy, C1-C8 alkoxy, C1-C8 branched or straight-chain alkyl, acyloxy, carbamoyl, amino, N-acylamino, nitro, halo, trihalomethyl, cyano, and carboxyl. Aryl moieties thus include, *e.g.* phenyl; substituted phenyl bearing one or more substituents selected from groups including: halo such as chloro or fluoro, hydroxy, C1-C6 alkyl, acyl, acyloxy, C1-C6 alkoxy (such as methoxy or ethoxy, including among others dialkoxyphenyl moieties such as 2,3-, 2,4-, 2,5-, 3,4- or 3,5-dimethoxy or diethoxy phenyl or such as methylenedioxyphenyl, or 3-methoxy-5-ethoxyphenyl; or trisubstituted phenyl, such as trialkoxy (*e.g.*, 3,4,5-trimethoxy or ethoxyphenyl), 3,5-dimethoxy-4-chlorophenyl, *etc.*), amino, -SO<sub>2</sub>NH<sub>2</sub>, -SO<sub>2</sub>NH(aliphatic), -SO<sub>2</sub>N(aliphatic)<sub>2</sub>, -O-aliphatic-COOH, and -O-aliphatic-NH<sub>2</sub> (which may contain one or two N-aliphatic or N-acyl substituents).

A "halo" substituent according to the present invention may be a fluoro, chloro, bromo or iodo substituent. Fluoro is often the preferred halogen.

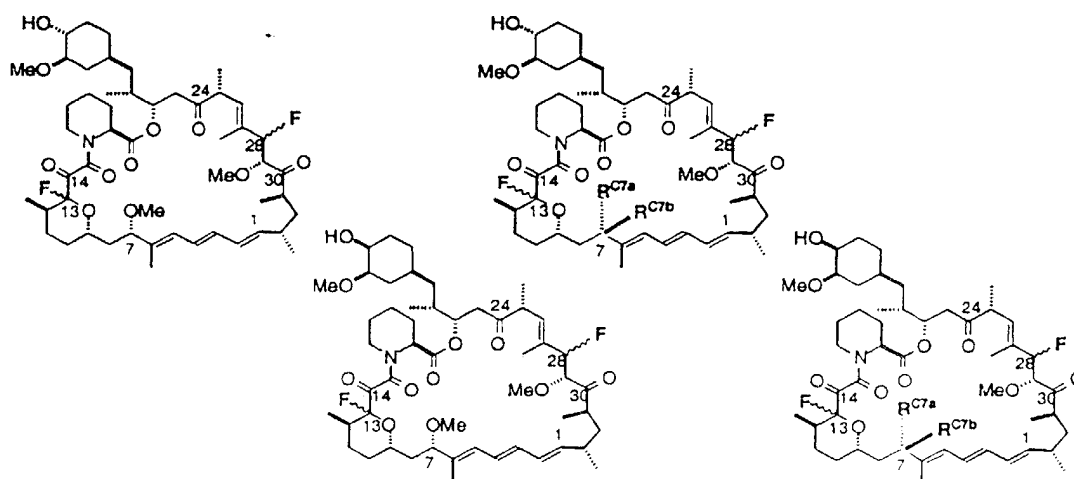
Compounds of formula II, exclusive of any compounds depicted in Table III, are of special interest and constitute an important class of novel compounds. Compounds of this class may differ from rapamycin with respect to one, two, three, four, five, six or seven substituent moieties. This class includes among others rapalogs with modifications, relative to rapamycin,

which  $R^{C30}$  and  $R^{C24}$  are both other than (=O). Of special interest are those C30.C24 rapalogs in which  $R^{C7a}$  is a moiety other than OMe. In certain embodiments of this subset,  $R^{C7a}$  and  $R^{C7b}$  are independently selected from -H, -OR<sup>1</sup>, -SR<sup>1</sup>, -OC(O)R<sup>1</sup> or -OC(O)NHR<sup>1</sup>, -NHR<sup>1</sup>, -NHC(O)R<sup>1</sup>, -NH-SO<sub>2</sub>-R<sup>1</sup> and -R<sup>2</sup>, where R<sup>2</sup> = substituted aryl or allyl or alkylaryl (e.g. benzyl or substituted benzyl), so long as one of  $R^{C7a}$  and  $R^{C7b}$  is H. In certain embodiments of this subset,  $R^{C30}$  and  $R^{C24}$  are both -OH, e.g. in the "S" configuration. In other embodiments  $R^{C30}$  and  $R^{C24}$  are independently selected from OR<sup>3</sup>. This subset includes among others all rapalogs in which  $R^{C30}$  and  $R^{C24}$  are OH and one of  $R^{C7a}$  and  $R^{C7b}$  comprises any of the replacement substituents at that position specified for formula II, including any of the C7 substituents identified in compounds of Tables II or III. This subset includes among others rapalogs which differ from rapamycin with respect to the moiety a. For instance, this subset includes compounds of the formula:



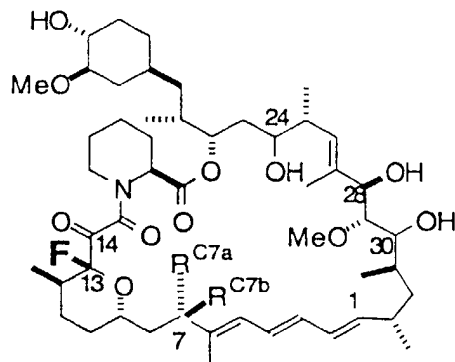
where at least one of  $R^{C7a}$  and  $R^{C7b}$  is other than -OMe. Alternative substituents for  $R^{C7a}$  and/or  $R^{C7b}$  are as disclosed elsewhere herein. Of special interest are compounds in which one of  $R^{C7a}$  and  $R^{C7b}$  is cyclic aliphatic, aryl, heterocyclic or heteroaryl, which may be optionally substituted. Other compounds within this subset include those in which one, two, three, four or five of the hydroxyl groups is epimerized, fluorinated, alkylated, acylated or otherwise modified via other ester, carbamate, carbonate or urea formation. An illustrative compound for example is the compound of formula III in which the hydroxyl group at C43 is epimerized and the hydroxyl groups at C28 and C30 are alkylated, acylated or linked via carbonate formation.

Another subset of improved rapalogs of special interest are those compounds of formula II in which one or both of  $R^{C13}$  and  $R^{C28}$  is F. In various embodiments of this subset, one, two, three,



The 13-fluoro rapalogs, including in particular 13-fluoro rapamycin and analogs and derivatives thereof containing various substituents which do not abolish immunosuppressive activity in rapamycin itself, are of interest as immunosuppressants.

An interesting intersection of some of the foregoing subsets of compounds is the set of improved rapalogs comprising compounds of formula II, or pharmaceutically acceptable derivatives thereof, in which  $R^{C24}$  and  $R^{C30}$  are both other than ( $=O$ ) and one or both of  $R^{C13}$  and  $R^{C28}$  is F. This set includes, *inter alia*, 24,30-tetrahydro-13-F rapalogs, 24,30-tetrahydro-28-F rapalogs and 24,30-tetrahydro-13,28-diF rapalogs, as well as C7 variants of any of the foregoing, in which  $R^{C7a}$  is other than OMe. A portion of that set is illustrated by the following structure, where  $R^{C7a}$  and  $R^{C7b}$  are as previously defined:



These compounds may be further derivatized, *e.g.*, by modifications at one or both of  $R^{C14}$  and  $R^{C43}$  relative to the C14 and C43 substituents in rapamycin itself.

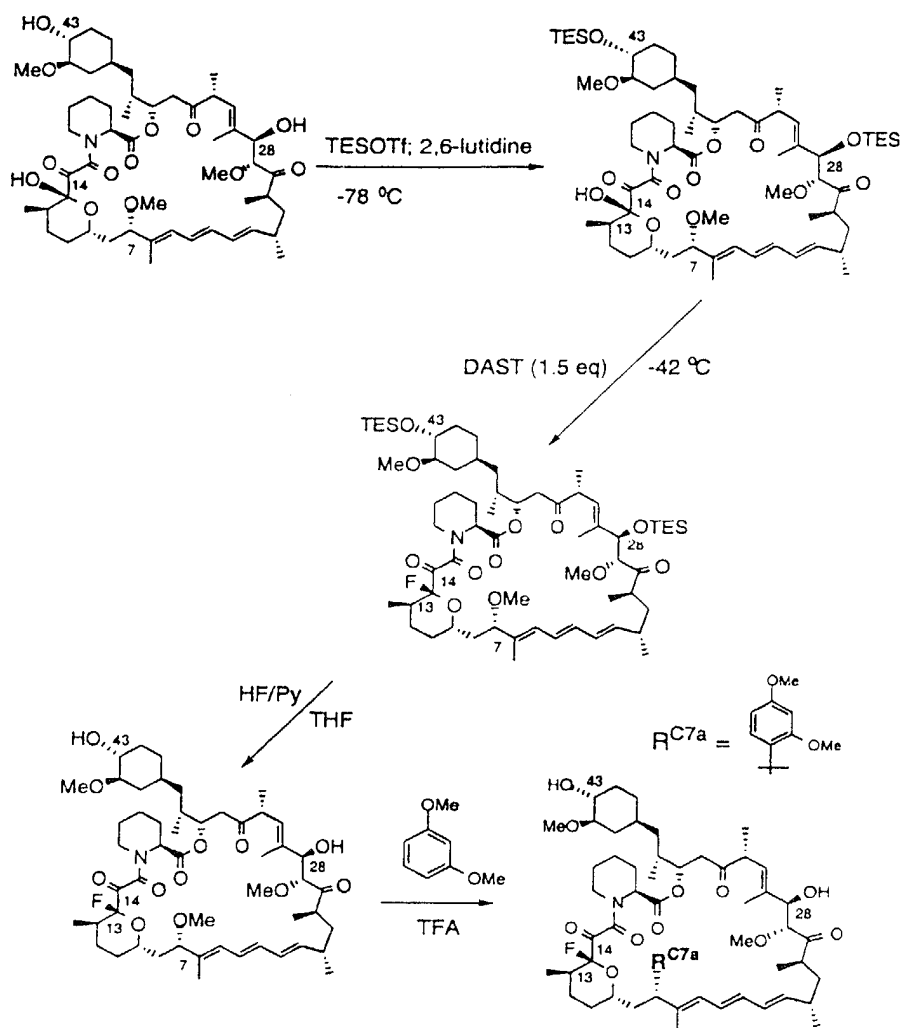
### Synthetic guidance

The production of rapamycin by fermentation and by total synthesis is known. The production of a number of rapalogs as fermentation products is also known. These include among  
 5 others rapalogs bearing alternative moieties to the characteristic cyclohexyl ring or pipercolate ring of rapamycin, as well as C7-desmethyl-rapamycin, C29-desmethyl-rapamycin and C29-desmethoxyrapamycin.

Methods and materials for effecting various chemical transformations of rapamycin and structurally related macrolides are known in the art, as are methods for obtaining rapamycin  
 10 and various rapalogs by fermentation. Many such chemical transformations of rapamycin and various rapalogs are disclosed in the patent documents identified in Table I, above, which serve to illustrate the level of skill and knowledge in the art of chemical synthesis and product recovery, purification and formulation which may be applied in practicing the subject invention. The following representative transformations and/or references which can be  
 15 employed to produce the desired rapalogs are illustrative:

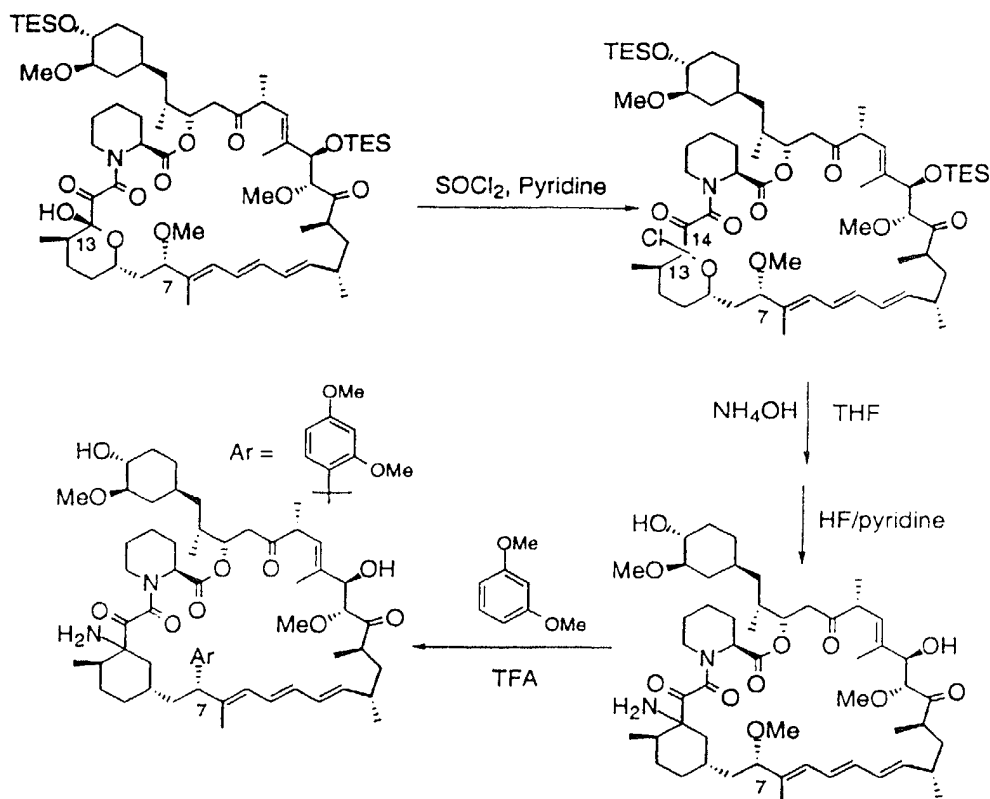
| ring position modified | literature reference  |
|------------------------|---|
| C7                     | Luengo, <i>et al.</i> JOC 59, 6512 (1995); Chem & Biol 2(7), 471-481 (1995)   |
| C-13                   | C13→F: protect C28 and C43, rxn at 0°   |
| C-14                   | Schubert, <i>et al.</i> Angew Chem Int Ed Engl 23, 167 (1984).  |
| C-20                   | Nelson, US Patent 5,387,680   |
| C-24                   | US Patent 5,373,014; 5,378,836<br>Lane, <i>et al.</i> Synthesis 1975, p136.   |
| C-30                   | Luengo <i>et al.</i> Tet. Lett. 35, 6469 (1994)   |
| various positions      | Or <i>et al.</i> , US Patent Nos. 5,527,907 and 5,583,139<br>Luengo, WO 94/02136; Cottens <i>et al.</i> , WO 95/16691 |

Approaches to the synthesis of the various fluoro and difluoro rapalogs are presented schematically below:



1. Smith, A. B. et al, *J. Am. Chem. Soc.* **1997**, 119, 962-973.
2. Middleton, W. J., *J. Org. Chem.*, **1975**, 40, 574-578.

notes: The tri-isopropylsilyl homolog, TIPs, may be used in place of the triethylsilyl protecting moiety, TES.  
The DAST reaction on the doubly protected rapamycin may be conducted at  $0^{\circ}\text{C}$  if desired.



See Donald, D. et al. WO 91/13889

Additionally, it is contemplated that rapalogs for use in this invention as well as intermediates for the production of such rapalogs may be prepared by directed biosynthesis, *e.g.* as described by Katz *et al.*, WO 93/13663 and by Cane *et al.*, WO 9702358. See also Khaw *et al.*, 1998, J. Bacteriology **180**(4):809-814 for additional biological methods.

Novel rapalogs of this invention may be prepared by one of ordinary skill in this art relying upon methods and materials known in the art as guided by the disclosure presented herein. For instance, methods and materials may be adapted from known methods set forth or referenced in the documents cited above, the full contents of which are incorporated herein by reference. Additional guidance and examples are provided herein by way of illustration and further guidance to the practitioner. It should be understood that the chemist of ordinary skill in this art would be readily able to make modifications to the foregoing, *e.g.* to add appropriate protecting groups to sensitive moieties during synthesis, followed by removal of the protecting groups when no longer needed or desired, and would be readily capable of determining other synthetic approaches.

selectively hybridizing to a DNA encoding that other protein, or would be capable of such hybridization but for the degeneracy of the genetic code.

FKBP fusion proteins of this invention, as well as FRB fusion proteins discussed in further detail below, may contain one or more copies of one or more different ligand binding domains and one or more copies of one or more action domains. The ligand binding domain(s) (i.e., FKBP and FRB domains) may be N-terminal, C-terminal, or interspersed with respect to the action domain(s). Embodiments involving multiple copies of a ligand binding domain usually have 2, 3 or 4 such copies. For example, an FKBP fusion protein may contain 2, 3 or 4 FKBP domains. The various domains of the FKBP fusion proteins (and of the FRB fusion proteins discussed below) are optionally separated by linking peptide regions which may be derived from one of the adjacent domains or may be heterologous.

Illustrative examples of FKBP fusion proteins useful in the practice of this invention include the FKBP fusion proteins disclosed in PCT/US94/01617 (Stanford & Harvard), PCT/US94/08008 (Stanford & Harvard), Spencer et al (supra), PCT/US95/10591 (ARIAD), PCT/US95/06722 (Mitotix, Inc.) and other references cited herein; the FKBP fusion proteins disclosed in the examples which follow; variants of any of the foregoing FKBP fusion proteins which contain up to 10 (preferably 1-5) amino acid insertions, deletions or substitutions in one or more of the FKBP domains and which are still capable of binding to rapamycin or to a rapalog; variants of any of the foregoing FKBP fusion proteins which contain one or more copies of an FKBP domain which is encoded by a DNA sequence capable of selectively hybridizing to a DNA sequence encoding a naturally occurring FKBP domain and which are still capable of binding to rapamycin or to a rapalog; variants of any of the foregoing in which one or more heterologous action domains are deleted, replaced or supplemented with a different heterologous action domain; variants of any of the foregoing FKBP fusion proteins which are capable of binding to rapamycin or a rapalog and which contain an FKBP domain derived from a non-human source; and variants of any of the foregoing FKBP fusion proteins which contain one or more amino acid residues corresponding to Tyr26, Phe36, Asp37, Arg42, Phe46, Phe48, Glu54, Val55, or Phe99 of human FKBP12 in which one or more of those amino acid residues is replaced by a different amino acid, the variant being capable of binding to rapamycin or a rapalog.

For instance, in a number of cases the FKBP fusion proteins comprise multiple copies of an FKBP domain containing amino acids 1-107 of human FKBP12, separated by the 2-amino acid linker Thr-Arg encoded by ACTAGA, the ligation product of DNAs digested respectively with the restriction endonucleases SpeI and XbaI. The following table provides illustrative subsets of mutant FKBP domains based on the foregoing FKBP12 sequence:



the FRB domain spans fewer than about 150 amino acids in length, and in some cases fewer than about 100 amino acids. One such region comprises a 133 amino acid region of human FRAP extending from Val2012 through Tyr2144. See Chiu et al, 1994, Proc. Natl. Acad. Sci. USA

91:12574-12578. An FRB region of particular interest spans Glu2025 through Gln2114 of human

5 FRAP and retains affinity for a FKBP12-rapamycin complex or for FKBP-rapalog complex. In some embodiments Q2214 is removed from the 90-amino acid sequence rendering this an 89-

amino acid FRB domain. The FRB peptide sequence may be modified to adjust the binding specificity, usually with replacement, insertion or deletion, of 10 or fewer, preferably 5 or fewer, amino acids. Such modifications are elected in certain embodiments to achieve a

10 preference towards formation of the complex comprising one or more molecules of the FKBP fusion protein, FRB fusion protein and an improved rapalog over formation of complexes of endogenous FKBP and FRAP proteins with the rapalog. Preferably that preference is at least one, and more preferably at least two, and even more preferably three, orders of magnitude (by any measure).

15 A recombinant DNA encoding such a protein will be capable of selectively hybridizing to a DNA encoding a FRAP species, or would be capable of such hybridization but for the degeneracy of the genetic code. Again, since these chimeric proteins contain an effector domain derived from another protein, e.g. Gal4, VP16, Fas, CD3 zeta chain, etc., the recombinant DNA encoding the chimeric protein will be capable of selectively hybridizing to a DNA encoding that other

20 protein, or would be capable of such hybridization but for the degeneracy of the genetic code.

Illustrative examples of FRB chimeras useful in the practice of this invention include those disclosed in the examples which follow, variants thereof in which one or more of the heterologous domains are replaced with alternative heterologous domains or supplemented with one or more additional heterologous domains, variants in which one or more of the FRB

25 domains is a domain of non-human peptide sequence origin (such as Tor 2 or Candida for example), and variants in which the FRB domain is modified by amino acid substitution, replacement or insertion as described herein, so long as the chimera is capable of binding to a complex formed by an FKBP protein and an improved rapalog of this invention. An illustrative FRB fusion protein contains one or more FRBs of at least 89-amino acids, containing a sequence

30 spanning at least residues 2025-2113 of human FRAP, separated by the linker Thr-Arg formed by ligation of SpeI-XbaI sites as mentioned previously. It should be appreciated that such restriction sites or linkers in any of the fusion proteins of this invention may be deleted, replaced or extended using conventional techniques such as site-directed mutagenesis.

or substituting a repressor domain (see PCT/US94/01617) in place of a transcription activation domain provides an analogous chimera useful for inhibiting transcription of a target gene. Composite DNA binding domains and DNA sequences to which they bind are disclosed in Pomerantz et al, 1995, supra, the contents of which are incorporated herein by reference. Such composite DNA binding domains may be used as DNA binding domains in the practice of this invention, together with a target gene construct containing the cognate DNA sequences to which the composite DBD binds.

In embodiments involving indirect activation of transcription, the heterologous domains of the chimeras are effector domains of signaling proteins which upon aggregation or multimerization trigger the activation of transcription under the control of a responsive promoter. For example, the signaling domain may be the intracellular domain of the zeta subunit of the T cell receptor, which upon aggregation, triggers transcription of a gene linked to the IL-2 promoter or a derivative thereof (e.g. iterated NF-AT binding sites).

In another aspect of the invention, the heterologous domains are protein domains which upon mutual association are capable of triggering cell death. Examples of such domains are the intracellular domains of the Fas antigen or of the TNF R1. Chimeric proteins containing a Fas domain can be designed and prepared by analogy to the disclosure of PCT/US94/01617.

#### Engineered receptor domains

As noted previously, the FKBP and FRB domains may contain peptide sequence selected from the peptide sequences of naturally occurring FKBP and FRB domains. Naturally occurring sequences include those of human FKBP12 and the FRB domain of human FRAP. Alternatively, the peptide sequences may be derived from such naturally occurring peptide sequences but contain generally up to 10, and preferably 1-5, mutations in one or both such peptide sequences. As disclosed in greater detail elsewhere herein, such mutations can confer a number of important features. For instance, an FKBP domain may be modified such that it is capable of binding an improved rapalog preferentially, i.e. at least one, preferably two, and even more preferably three or four or more orders of magnitude more effectively, with respect to rapalog binding by the unmodified FKBP domain. An FRB domain may be modified such that it is capable of binding a (modified or unmodified) FKBP:rapalog complex preferentially, i.e. at least one, preferably two, and even more preferably three orders of magnitude more effectively, with respect to the unmodified FRB domain. FKBP and FRB domains may be modified such that they are capable of forming a tripartite complex with an improved rapalog, preferentially, i.e. at

more rapalog or rapamycin substituents. A collection of polypeptides containing FKBP domains randomized at the identified positions (such as are noted in the foregoing paragraph) is prepared e.g. using conventional synthetic or genetic methods. Such a collection represents a set of FKBP domains containing replacement amino acids at one or more of such positions. The collection is screened and FKBP variants are selected which possess the desired rapalog binding properties. In general, randomizing several residues simultaneously is expected to yield compensating mutants of higher affinity and specificity for a given bumped rapalog as it maximizes the likelihood of beneficial cooperative interactions between sidechains. Techniques for preparing libraries randomized at discrete positions are known and include primer-directed mutagenesis using degenerate oligonucleotides, PCR with degenerate oligonucleotides, and cassette mutagenesis with degenerate oligonucleotides (see for example Lowman, H.B, and Wells, J.A. *Methods: Comp. Methods Enzymol.* 1991. 3, 205-216; Dennis, M.S. and Lazarus, R.A. 1994. *J. Biol. Chem.* 269, 22129-22136; and references therein).

We further contemplate that in many cases, randomization of only the few residues in or near direct contact with a given position in rapamycin may not completely explore all the possible variations in FKBP conformation that could optimally accommodate a rapalog substituent (bump). Thus the construction is also envisaged of unbiased libraries containing random substitutions that are not based on structural considerations, to identify subtle mutations or combinations thereof that confer preferential binding to bumped rapalogs. Several suitable mutagenesis schemes have been described, including alanine-scanning mutagenesis (Cunningham and Wells (1989) *Science* 244, 1081-1085), PCR misincorporation mutagenesis (see eg. Cadwell and Joyce, 1992, *PCR Meth. Applic.* 2, 28-33), and 'DNA shuffling' (Stemmer, 1994, *Nature* 370, 389-391 and Crameri et al, 1996, *Nature Medicine* 2, 100-103). These techniques produce libraries of random mutants, or sets of single mutants, that are then searched by screening or selection approaches.

In many cases, an effective strategy to identify the best mutants for preferential binding of a given bump is a combination of structure-based and unbiased approaches. See Clackson and Wells, 1994, *Trends Biotechnology* 12, 173-184 (review). For example we contemplate the construction of libraries in which key contact residues are randomized by PCR with degenerate oligonucleotides, but with amplification performed using error-promoting conditions to introduce further mutations at random sites. A further example is the combination of component DNA fragments from structure-based and unbiased random libraries using DNA shuffling.

Screening of libraries for desirable mutations may be performed by use of a yeast 2-hybrid system (Fields and Song (1989) *Nature* 340, 245-246). For example, an FRB-VP16 fusion may be introduced into one vector, and a library of randomized FKBP sequences cloned into a separate

phage, display on plasmids, display on baculovirus). Furthermore, selection and screening strategies can also be used to improve other properties of benefit in the application of this invention, such as enhanced stability in vivo. For a review see Clackson, T. & Wells, J.A. 1994. Trends Biotechnol. 12, 173-184.

## 5 (b) FRAP

Similar considerations apply to the generation of mutant FRB domains which bind preferentially to improved rapalogs containing modifications (i.e., are 'bumped') relative to rapamycin in the FRAP-binding portion of the macrocycle. For example, one may obtain preferential binding using rapalogs bearing substituents other than -OMe at the C7 position with FRBs based on the human FRAP FRB peptide sequence but bearing amino acid substitutions for one of more of the residues Tyr2038, Phe2039, Thr2098, Gln2099, Trp2101 and Asp2102. Exemplary mutations include Y2038H, Y2038L, Y2038V, Y2038A, F2039H, F2039L, F2039A, F2039V, D2102A, T2098A, T2098N, and T2098S. Rapalogs bearing substituents other than -OH at C28 and/or substituents other than =O at C30 may be used to obtain preferential binding to FRAP proteins bearing an amino acid substitution for Glu2032. Exemplary mutations include E2032A and E2032S. Proteins comprising an FRB containing one or more amino acid replacements at the foregoing positions, libraries of proteins or peptides randomized at those positions (i.e., containing various substituted amino acids at those residues), libraries randomizing the entire protein domain, or combinations of these sets of mutants are made using the procedures described above to identify mutant FRAPs that bind preferentially to bumped rapalogs.

The affinity of candidate mutant FRBs for the complex of an FKBP protein complexed with a rapalog may be assayed by a number of techniques; for example binding of in vitro translated FRB mutants to GST-FKBP in the presence of drug (Chen et al. 1995. Proc. Natl. Acad. Sci. USA 92, 4947-4951); or ability to participate in a rapalog-dependent transcriptionally active complex with an appropriate FKBP fusion protein in a yeast two-hybrid assay.

FRB mutants with desired binding properties may be isolated from libraries displayed on phage using a variety of sorting strategies. For example, a rapalog is mixed with the library phage pool in solution in the presence of recombinant FKBP tagged with an affinity handle (for example a hexa-histidine tag, or GST), and the resultant complexes are captured on the appropriate affinity matrix to enrich for phage displaying FRAP harboring complementary mutations.

An additional feature of the FRB fusion protein that may vary in the various embodiments of this invention is the exact sequence of the FRB domain used. In some applications it may be preferred to use portions of an FRB which are larger than the minimal (89 amino acid) FRB.

used (i) to identify amino acid substitutions, deletions or insertions to an FKBP domain which measurably diminish the domain's ability to form the tripartite complex with a given rapalog and the endogenous FRB; (ii) to identify amino acid substitutions, deletions or insertions to an FRB domain which measurably diminish the domain's ability to form the tripartite complex with a given rapalog and the endogenous FKBP; and (iii) to select and/or otherwise identify compensating mutation(s) in the partner protein. As examples of suitable mutant FKBP's with diminished effectiveness in tripartite complex formation, we include mammalian, preferably human FKBP in which one or both of His87 and Ile90 are replaced with amino acids such as Arg, Trp, Phe, Tyr or Lys which contain bulky side chain groups; FRB domains, preferably containing mammalian, and more preferably of human, peptide sequence may then be mutated as described above to generate complementary variants which are capable of forming a tripartite complex with the mutant FKBP and a given rapalog. Illustrative FRB mutations which may be useful with H87W or H87R hFKBP12s include human FRBs in which Y2038 is replaced by V, S, A or L; F2039 is replaced by A; and/or R2042 is replaced by L, A or S. Illustrative FRB mutations which may be useful with I90W or I90R hFKBP12s include human FRBs in which K2095 is replaced with L, S, A or T.

Additionally, in optimizing the receptor domains of this invention, it should be appreciated that immunogenicity of a polypeptide sequence is thought to require the binding of peptides by MHC proteins and the recognition of the presented peptides as foreign by endogenous T-cell receptors. It may be preferable, at least in human gene therapy applications, to tailor a given foreign peptide sequence, including junction peptide sequences, to minimize the probability of its being immunologically presented in humans. For example, peptide binding to human MHC class I molecules has strict requirements for certain residues at key 'anchor' positions in the bound peptide: eg. HLA-A2 requires leucine, methionine or isoleucine at position 2 and leucine or valine at the C-terminus (for review see Stern and Wiley (1994) Structure 2, 145-251). Thus in engineering proteins in the practice of this invention, this periodicity of these residues is preferably avoided, especially in human gene therapy applications. The foregoing applies to all protein engineering aspects of the invention, including without limitation the engineering of point mutations into receptor domains, and to the choice or design of boundaries between the various protein domains.

#### Other components, design features and applications

The chimeric proteins may contain as a heterologous domain a cellular localization domain such as a membrane retention domain. See e.g. PCT/US94/01617, especially pages 26-27.

Briefly, a membrane retention domain can be isolated from any convenient membrane-bound

same species as the host cell. Thus, for genetic engineering of human cells, it is often preferred that the heterologous domains (as well as the FKBP and FRB domains) be of human origin, rather than of bacterial, yeast or other non-human source.

We also note that epitope tags may also be incorporated into chimeric proteins of this invention to permit convenient detection.

#### Tissue-specific or cell-type specific expression

It will be preferred in certain embodiments, that the chimeric proteins be expressed in a cell-specific or tissue-specific manner. Such specificity of expression may be achieved by operably linking one or more of the DNA sequences encoding the chimeric protein(s) to a cell-type specific transcriptional regulatory sequence (e.g. promoter/enhancer). Numerous cell-type specific transcriptional regulatory sequences are known. Others may be obtained from genes which are expressed in a cell-specific manner. See e.g. PCT/US95/10591, especially pp. 36-37.

For example, constructs for expressing the chimeric proteins may contain regulatory sequences derived from known genes for specific expression in selected tissues. Representative examples are tabulated below:

| Tissue                        | Gene           | Reference  |
|-------------------------------|----------------|--|
| lens                          | g2-crystallin  | Breitman, M.L., Clapoff, S., Rossant, J., Tsui, L.C., Golde, L.M., Maxwell, I.H., Bernstein, A. (1987) Genetic Ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. <i>Science</i> 238: 1563-1565 |
|                               | aA-crystallin  | Landel, C.P., Zhao, J., Bok, D., Evans, G.A. (1988) Lens-specific expression of a recombinant ricin induces developmental defects in the eyes of transgenic mice. <i>Genes Dev.</i> 2: 1168-1178                                     |
|                               |                | Kaur, S., Key, B., Stock, J., McNeish, J.D., Akesson, R., Potter, S.S. (1989) Targeted ablation of alpha-crystallin-synthesizing cells produces lens-deficient eyes in transgenic mice. <i>Development</i> 105: 613-619              |
| pituitary - somatrophic cells | Growth hormone | Behringer, R.R., Mathews, L.S., Palmiter, R.D., Brinster, R.L. (1988) Dwarf mice produced by genetic ablation of growth hormone-expressing cells. <i>Genes Dev.</i> 2: 453-461 .../...   |

|       |  |  |
|-------|--|--|
| liver | tyrosine<br>aminotransfer-<br>ase, albumin,<br>apolipoproteins |  |
|-------|--|--|

### Target Gene Constructs

In embodiments of the invention in which the chimeric proteins are designed such that their multimerization activates transcription of a target gene, an appropriate target gene construct is also used in the engineered cells. Appropriate target gene constructs are those containing a target gene and a cognate transcriptional control element such as a promoter and/or enhancer which is responsive to the multimerization of the chimeric proteins. In embodiments involving direct activation of transcription, that responsiveness may be achieved by the presence in the target gene construct of one or more DNA sequences recognized by the DNA-binding domain of a chimeric protein of this invention (i.e., a DNA sequence to which the chimeric protein binds). In embodiments involving indirect activation of transcription, responsiveness may be achieved by the presence in the target gene construct of a promoter and/or enhancer sequence which is activated by an intracellular signal generated by multimerization of the chimeric proteins. For example, where the chimeric proteins contain the TCR zeta chain intracellular domain, the target gene is linked to and under the expression control of the IL-2 promoter region.

This invention also provides target DNA constructs containing (a) a cognate DNA sequence, e.g. to which a DNA-binding chimeric protein of this invention is capable of binding (or which is susceptible to indirect activation as discussed above), and (b) flanking DNA sequence from the locus of a desired target gene endogenous to the host cells. These constructs permit homologous recombination of the cognate DNA sequence into a host cell in association with an endogenous target gene. In other embodiments the construct contains a desired gene and flanking DNA sequence from a target locus permitting the homologous recombination of the target gene into the desired locus. Such a target construct may also contain the cognate DNA sequence, or the cognate DNA sequence may be provided by the locus.

The target gene in any of the foregoing embodiments may encode for example a surface membrane protein (such as a receptor protein), a secreted protein, a cytoplasmic protein, a nuclear protein, a recombinase such as Cre, a ribozyme or an antisense RNA. See PCT/US94/01617 for general design and construction details and for various applications including gene therapy and see PCT/US95/10591 regarding applications to animal models of disease.

biochemical analysis of the encoded chimera, it may be desirable to construct plasmids that direct the expression of the protein in bacteria or in reticulocyte-lysate systems. For use in the production of proteins in mammalian cells, the protein-encoding sequence is introduced into an expression vector that directs expression in these cells. Expression vectors suitable for such uses are well known in the art. Various sorts of such vectors are commercially available.

Constructs encoding the chimeric proteins and target genes of this invention can be introduced into the cells as one or more DNA molecules or constructs, in many cases in association with one or more markers to allow for selection of host cells which contain the construct(s). The construct(s) once completed and demonstrated to have the appropriate sequences may then be introduced into a host cell by any convenient means. The constructs may be incorporated into vectors capable of episomal replication (e.g. BPV or EBV vectors) or into vectors designed for integration into the host cells' chromosomes. The constructs may be integrated and packaged into non-replicating, defective viral genomes like Adenovirus, Adeno-associated virus (AAV), or Herpes simplex virus (HSV) or others, including retroviral vectors, for infection or transduction into cells. Viral delivery systems are discussed in greater detail below. Alternatively, the construct may be introduced by protoplast fusion, electro-poration, biolistics, calcium phosphate transfection, lipofection, microinjection of DNA or the like. The host cells will in some cases be grown and expanded in culture before introduction of the construct(s), followed by the appropriate treatment for introduction of the construct(s) and integration of the construct(s). The cells will then be expanded and screened by virtue of a marker present in the constructs. Various markers which may be used successfully include hp<sup>r</sup>t, neomycin resistance, thymidine kinase, hygromycin resistance, etc., and various cell-surface markers such as Tac, CD8, CD3, Thy1 and the NGF receptor.

In some instances, one may have a target site for homologous recombination, where it is desired that a construct be integrated at a particular locus. For example, one can delete and/or replace an endogenous gene (at the same locus or elsewhere) with a recombinant target construct of this invention. For homologous recombination, one may generally use either  $\Omega$  or O-vectors. See, for example, Thomas and Capecchi, Cell (1987) 51, 503-512; Mansour, et al., Nature (1988) 336, 348-352; and Joyner, et al., Nature (1989) 338, 153-156.

The constructs may be introduced as a single DNA molecule encoding all of the genes, or different DNA molecules having one or more genes. The constructs may be introduced simultaneously or consecutively, each with the same or different markers.

Vectors containing useful elements such as bacterial or yeast origins of replication, selectable and/or amplifiable markers, promoter/enhancer elements for expression in procaryotes or eucaryotes, and mammalian expression control elements, etc. which may be used



into the culture medium, which can then be used to infect cells in culture. Since retroviruses are unable to infect non-dividing cells, they have been used primarily for ex vivo gene therapy.

### AAV Vectors

5 Adeno-associated virus (AAV)-based vectors are of general interest as a delivery vehicle to various tissues, including muscle and lung. AAV vectors infect cells and stably integrate into the cellular genome with high frequency. AAV can infect and integrate into growth-arrested cells (such as the pulmonary epithelium), and is non-pathogenic.

The AAV-based expression vector to be used typically includes the 145 nucleotide AAV  
10 inverted terminal repeats (ITRs) flanking a restriction site that can be used for subcloning or the transgene, either directly using the restriction site available, or by excision of the transgene with restriction enzymes followed by blunting of the ends, ligation of appropriate DNA linkers, restriction digestion, and ligation into the site between the ITRs. The capacity of AAV vectors is about 4.4 kb. The following proteins have been expressed using various AAV-based vectors,  
15 and a variety of promoter/enhancers: neomycin phosphotransferase, chloramphenicol acetyl transferase, Fanconi's anemia gene, cystic fibrosis transmembrane conductance regulator, and granulocyte macrophage colony-stimulating factor (Kotin, R.M., Human Gene Therapy 5:793-801, 1994, Table I). A transgene incorporating the various DNA constructs of this invention can similarly be included in an AAV-based vector. As an alternative to inclusion of a  
20 constitutive promoter such as CMV to drive expression of the recombinant DNA encoding the fusion protein(s), an AAV promoter can be used (ITR itself or AAV p5 (Flotte, et al. J. Biol. Chem. 268:3781-3790, 1993)).

Such a vector can be packaged into AAV virions by reported methods. For example, a human cell line such as 293 can be co-transfected with the AAV-based expression vector and another  
25 plasmid containing open reading frames encoding AAV rep and cap under the control of endogenous AAV promoters or a heterologous promoter. In the absence of helper virus, the rep proteins Rep68 and Rep78 prevent accumulation of the replicative form, but upon superinfection with adenovirus or herpes virus, these proteins permit replication from the ITRs (present only in the construct containing the transgene) and expression of the viral capsid proteins. This  
30 system results in packaging of the transgene DNA into AAV virions (Carter, B.J., Current Opinion in Biotechnology 3:533-539, 1992; Kotin, R.M, Human Gene Therapy 5:793-801, 1994)). Methods to improve the titer of AAV can also be used to express the transgene in an AAV virion. Such strategies include, but are not limited to: stable expression of the ITR-flanked transgene in a cell line followed by transfection with a second plasmid to direct viral packaging; use of a cell

delivery protocol, by the same methods (restriction digest, linker ligation or filling in of ends, and ligation) used to insert the CFTR or other genes into the vectors. Hybrid Adenovirus-AAV vectors represented by an adenovirus capsid containing selected portions of the adenovirus sequence, 5' and 3' AAV ITR sequences flanking the transgene and other conventional vector regulatory elements may also be used. See e.g. Wilson et al, International Patent Application Publication No. WO 96/13598. For additional detailed guidance on adenovirus and hybrid adenovirus-AAV technology which may be useful in the practice of the subject invention, including methods and materials for the incorporation of a transgene, the propagation and purification of recombinant virus containing the transgene, and its use in transfecting cells and mammals, see also Wilson et al, WO 94/28938, WO 96/13597 and WO 96/26285, and references cited therein.

Generally the DNA or viral particles are transferred to a biologically compatible solution or pharmaceutically acceptable delivery vehicle, such as sterile saline, or other aqueous or non-aqueous isotonic sterile injection solutions or suspensions, numerous examples of which are well known in the art, including Ringer's, phosphate buffered saline, or other similar vehicles.

Preferably, in gene therapy applications, the DNA or recombinant virus is administered in sufficient amounts to transfect cells at a level providing therapeutic benefit without undue adverse effects. Optimal dosages of DNA or virus depends on a variety of factors, as discussed elsewhere, and may thus vary somewhat from patient to patient. Again, therapeutically effective doses of viruses are considered to be in the range of about 20 to about 50 ml of saline solution containing concentrations of from about  $1 \times 10^7$  to about  $1 \times 10^{10}$  pfu of virus/ml, e.g. from  $1 \times 10^8$  to  $1 \times 10^9$  pfu of virus/ml.

## Host Cells

This invention is particularly useful for the engineering of animal cells and in applications involving the use of such engineered animal cells. The animal cells may be insect, worm or mammalian cells. While various mammalian cells may be used, including, by way of example, equine, bovine, ovine, canine, feline, murine, and non-human primate cells, human cells are of particular interest. Among the various species, various types of cells may be used, such as hematopoietic, neural, glial, mesenchymal, cutaneous, mucosal, stromal, muscle (including smooth muscle cells), spleen, reticulo-endothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, fibroblast, and other cell types. Of particular interest are hematopoietic cells, which may include any of the nucleated cells which may be involved with

organism or patient for the production of a therapeutic protein. See e.g. Hguyen et al, Tissue Implant Systems and Methods for Sustaining viable High Cell Densities within a Host, US Patent No. 5,314,471 (Baxter International, Inc.); Uludag and Sefton, 1993, J Biomed. Mater. Res. 27(10):1213-24 (HepG2 cells/hydroxyethyl methacrylate-methyl methacrylate membranes);

- 5 Chang et al, 1993, Hum Gene Ther 4(4):433-40 (mouse Ltk- cells expressing hGH/immunoprotective perm-selective alginate microcapsules; Reddy et al, 1993, J Infect Dis 168(4):1082-3 (alginate); Tai and Sun, 1993, FASEB J 7(11):1061-9 (mouse fibroblasts expressing hGH/alginate-poly-L-lysine-alginate membrane); Ao et al, 1995, Transplantation Proc. 27(6):3349, 3350 (alginate); Rajotte et al, 1995, Transplantation Proc. 27(6):3389 (alginate);
- 10 Lakey et al, 1995, Transplantation Proc. 27(6):3266 (alginate); Korbitt et al, 1995, Transplantation Proc. 27(6):3212 (alginate); Dorian et al, US Patent No. 5,429,821 (alginate); Emerich et al, 1993, Exp Neurol 122(1):37-47 (polymer-encapsulated PC12 cells); Sagen et al, 1993, J Neurosci 13(6):2415-23 (bovine chromaffin cells encapsulated in semipermeable polymer membrane and implanted into rat spinal subarachnoid space); Aebischer et al, 1994, Exp Neurol 126(2):151-8 (polymer-encapsulated rat PC12 cells implanted into monkeys; see also Aebischer, WO 92/19595); Savelkoul et al, 1994, J Immunol Methods 170(2):185-96 (encapsulated hybridomas producing antibodies; encapsulated transfected cell lines expressing various cytokines); Winn et al, 1994, PNAS USA 91(6):2324-8 (engineered BHK cells expressing human nerve growth factor encapsulated in an immunoisolation polymeric device and transplanted into
- 20 rats); Emerich et al, 1994, Prog Neuropsychopharmacol Biol Psychiatry 18(5):935-46 (polymer-encapsulated PC12 cells implanted into rats); Kordower et al, 1994, PNAS USA 91(23):10898-902 (polymer-encapsulated engineered BHK cells expressing hNGF implanted into monkeys) and Butler et al WO 95/04521 (encapsulated device). The cells may then be introduced in encapsulated form into an animal host, preferably a mammal and more preferably a human
- 25 subject in need thereof. Preferably the encapsulating material is semipermeable, permitting release into the host of secreted proteins produced by the encapsulated cells. In many embodiments the semipermeable encapsulation renders the encapsulated cells immunologically isolated from the host organism in which the encapsulated cells are introduced. In those embodiments the cells to be encapsulated may express one or more chimeric proteins containing
- 30 component domains derived from proteins of the host species and/or from viral proteins or proteins from species other than the host species. For example in such cases the chimeras may contain elements derived from GAL4 and VP16. The cells may be derived from one or more individuals other than the recipient and may be derived from a species other than that of the recipient organism or patient.

- 35 Instead of ex vivo modification of the cells, in many situations one may wish to modify cells in vivo. For this purpose, various techniques have been developed for modification of target

Corresponding cell-based assays may also be conducted in engineered yeast cells. See e.g. WO 95/33052 (Berlin et al).

It will often be preferred that the rapalogs of this invention be physiologically acceptable (i.e., lack undue toxicity toward the cell or organism with which it is to be used), can be taken orally by animals (i.e., is orally active in applications in whole animals, including gene therapy), and/or can cross cellular and other membranes, as necessary for a particular application.

In addition, preferred rapalogs are those which bind preferentially to mutant immunophilins (by way of non-limiting example, a human FKBP in which Phe36 is replaced with a different amino acid, preferably an amino acid with a less bulky R group such as valine or alanine) over native or naturally-occurring immunophilins. For example, such compounds may bind preferentially to mutant FKBP's at least an order of magnitude better than they bind to human FKBP12, and in some cases may bind to mutant FKBP's greater than 2 or even 3 or more orders of magnitude better than they do to human FKBP12, as determined by any scientifically valid or art-accepted assay methodology.

Binding affinities of various rapalogs of this invention with respect to human FKBP12, variants thereof or other immunophilin proteins may be determined by adaptation of known methods used in the case of FKBP. For instance, the practitioner may measure the ability of a compound of this invention to compete with the binding of a known ligand to the protein of interest. See e.g. Sierkierka et al, 1989, Nature 341, 755-757 (test compound competes with binding of labeled FK506 derivative to FKBP).

One set of preferred rapalogs of this invention which binds, to human FKBP12, to a mutant thereof as discussed above, or to a fusion protein containing such FKBP domains, with a K<sub>d</sub> value below about 200 nM, more preferably below about 50 nM, even more preferably below about 10 nM, and even more preferably below about 1 nM, as measured by direct binding measurement (e.g. fluorescence quenching), competition binding measurement (e.g. versus FK506), inhibition of FKBP enzyme activity (rotamase), or other assay methodology. In one subset of such compounds, the FKBP domain is one in which phenylalanine at position 36 has been replaced with an amino acid having a less bulky side chain, e.g. alanine, valine, methionine or serine.

A Competitive Binding FP Assay is described in detail in the examples which follow. That assay permits the in vitro measurement of an IC<sub>50</sub> value for a given compound which reflects its ability to bind to an FKBP protein in competition with a labeled FKBP ligand, such as, for example, FK506.

express a detectable product. The cells also contain and are capable of expressing DNAs encoding chimeric proteins comprising one or more immunophilin-derived ligand binding domains and one or more effector domains, such as the intracellular domain of FAS, capable, upon multimerization, of triggering cell death. The design and preparation of illustrative components and their use in so engineering cells is described in WO95/02684. See also WO96/41865. The cells are maintained or cultured in a culture medium permitting cell growth or continued viability. The cells or medium are assayed for the presence of the constitutive cellular product, and a base-line level of reporter is thus established. One may use cells engineered for constitutive production of hGH or any other conveniently detectable product to serve as the reporter. The compound to be tested is added to the medium, the cells are incubated, and the cell lysate or medium is tested for the presence of reporter at one or more time points. Decrease in reporter production indicates cell death, an indirect measure of multimerization of the fusion proteins.

Another preferred class of compounds of this invention are those which are capable of inducing a detectable signal in such an FKBP/FRB-based apoptosis assay. Preferably, the FKBP domain is an FKBP domain other than wild-type human FKBP12. In some cases, the FKBP domain is modified, as discussed above. Also preferably, the FRB domain is an FRB domain other than wild-type FRB from human FRAP. In some cases, the FRB domain is modified at position 2098, as described above.

Conducting such assays permits the practitioner to select rapalogs possessing the desired IC50 values and/or binding preference for a mutant FKBP over wild-type human FKBP12. The Competitive Binding FP Assay permits one to select monomers or rapalogs which possess the desired IC50 values and/or binding preference for a mutant FKBP or wild-type FKBP relative to a control, such as FK506.

## Applications

The rapalogs can be used as described in WO94/18317, WO95/02684, WO96/20951, WO95/41865, e.g. to regulatably activate the transcription of a desired gene, delete a target gene, actuate apoptosis, or trigger other biological events in engineered cells growing in culture or in whole organisms, including in gene therapy applications. The following are non-limiting examples of applications of the subject invention.

**1. Regulated gene therapy.** In many instances, the ability to switch a therapeutic gene on and off at will or the ability to titrate expression with precision are important for therapeutic efficacy. This invention is particularly well suited for achieving regulated expression of a

therapeutic effect, an antagonist to the dimerizing agent can be administered in any convenient way, particularly intravascularly, if a rapid reversal is desired. Alternatively, one may provide for the presence of an inactivation domain (or transcriptional silencer) with a ligand binding domain. In another approach, cells may be eliminated through apoptosis via signalling through Fas or TNF receptor as described elsewhere. See International Patent Applications PCT/US94/01617 and PCT/US94/08008.

The particular dosage of the improved rapalog for any application may be determined in accordance with the procedures used for therapeutic dosage monitoring, where maintenance of a particular level of expression is desired over an extended period of times, for example, greater than about two weeks, or where there is repetitive therapy, with individual or repeated doses of improved rapalog over short periods of time, with extended intervals, for example, two weeks or more. A dose of the improved rapalog within a predetermined range would be given and monitored for response, so as to obtain a time-expression level relationship, as well as observing therapeutic response. Depending on the levels observed during the time period and the therapeutic response, one could provide a larger or smaller dose the next time, following the response. This process would be iteratively repeated until one obtained a dosage within the therapeutic range. Where the improved rapalog is chronically administered, once the maintenance dosage of the improved rapalog is determined, one could then do assays at extended intervals to be assured that the cellular system is providing the appropriate response and level of the expression product.

It should be appreciated that the system is subject to many variables, such as the cellular response to the improved rapalog, the efficiency of expression and, as appropriate, the level of secretion, the activity of the expression product, the particular need of the patient, which may vary with time and circumstances, the rate of loss of the cellular activity as a result of loss of cells or expression activity of individual cells, and the like.

**2. Production of recombinant proteins and viruses.** Production of recombinant therapeutic proteins for commercial and investigational purposes is often achieved through the use of mammalian cell lines engineered to express the protein at high level. The use of mammalian cells, rather than bacteria or yeast, is indicated where the proper function of the protein requires post-translational modifications not generally performed by heterologous cells. Examples of proteins produced commercially this way include erythropoietin, tissue plasminogen activator, clotting factors such as Factor VIII:c, antibodies, etc. The cost of producing proteins in this fashion is directly related to the level of expression achieved in the engineered cells. A second limitation on the production of such proteins is toxicity to the host

chimeric proteins. Alternatively, the target gene construct may contain a cloning site for insertion of a desired target gene by the practitioner. Such kits may also contain a sample of a dimerizing agent capable of dimerizing the two recombinant proteins and activating transcription of the target gene.

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#### Formulations, dosage and administration

By virtue of its capacity to promote protein-protein interactions, a rapalog of this invention may be used in pharmaceutical compositions and methods for promoting formation of complexes of chimeric proteins of this invention in a human or non-human mammal containing genetically engineered cells of this invention.

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The preferred method of such treatment or prevention is by administering to the mammal an effective amount of the compound to promote measurable formation of such complexes in the engineered cells, or preferably, to promote measurable actuation of the desired biological event triggered by such complexation, e.g. transcription of a target gene, apoptosis of engineered cells, etc.

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#### Therapeutic/Prophylactic Administration & Pharmaceutical Compositions

The rapalogs can exist in free form or, where appropriate, in salt form. Pharmaceutically acceptable salts of many types of compounds and their preparation are well-known to those of skill in the art. The pharmaceutically acceptable salts of compounds of this invention include the conventional non-toxic salts or the quaternary ammonium salts of such compounds which are formed, for example, from inorganic or organic acids or bases.

20

The compounds of the invention may form hydrates or solvates. It is known to those of skill in the art that charged compounds form hydrated species when lyophilized with water, or form solvated species when concentrated in a solution with an appropriate organic solvent.

25

This invention also relates to pharmaceutical compositions comprising a therapeutically (or prophylactically) effective amount of the compound, and one or more pharmaceutically acceptable carriers and/or other excipients. Carriers include e.g. saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof, and are discussed in greater detail below. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral

30

The carrier or excipient may include time delay material well known to the art, such as glyceryl monostearate or glyceryl distearate along or with a wax, ethylcellulose, hydroxypropylmethylcellulose, methylmethacrylate and the like. When formulated for oral administration, 0.01% Tween 80 in PHOSAL PG-50 (phospholipid concentrate with  
5 1,2-propylene glycol, A. Nattermann & Cie. GmbH) has been recognized as providing an acceptable oral formulation for other compounds, and may be adapted to formulations for various compounds of this invention.

A wide variety of pharmaceutical forms can be employed. If a solid carrier is used, the preparation can be tableted, placed in a hard gelatin capsule in powder or pellet form or in the  
10 form of a troche or lozenge. The amount of solid carrier will vary widely but preferably will be from about 25 mg to about 1 g. If a liquid carrier is used, the preparation will be in the form of a syrup, emulsion, soft gelatin capsule, sterile injectable solution or suspension in an ampule or vial or nonaqueous liquid suspension.

To obtain a stable water soluble dosage form, a pharmaceutically acceptable salt of the  
15 multimerizer may be dissolved in an aqueous solution of an organic or inorganic acid, such as a 0.3M solution of succinic acid or citric acid. Alternatively, acidic derivatives can be dissolved in suitable basic solutions. If a soluble salt form is not available, the compound is dissolved in a suitable cosolvent or combinations thereof. Examples of such suitable cosolvents include, but are not limited to, alcohol, propylene glycol, polyethylene glycol 300, polysorbate 80, glycerin,  
20 polyoxyethylated fatty acids, fatty alcohols or glycerin hydroxy fatty acids esters and the like in concentrations ranging from 0-60% of the total volume.

Various delivery systems are known and can be used to administer the multimerizer, or the various formulations thereof, including tablets, capsules, injectable solutions, encapsulation in liposomes, microparticles, microcapsules, *etc.* Methods of introduction include but are not  
25 limited to dermal, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, pulmonary, epidural, ocular and (as is usually preferred) oral routes. The compound may be administered by any convenient or otherwise appropriate route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically  
30 active agents. Administration can be systemic or local. For treatment or prophylaxis of nasal, bronchial or pulmonary conditions, preferred routes of administration are oral, nasal or via a bronchial aerosol or nebulizer.

In certain embodiments, it may be desirable to administer the compound locally to an area in need of treatment; this may be achieved by, for example, and not by way of limitation, local  
35 infusion during surgery, topical application, by injection, by means of a catheter, by means of a



illustrative formulation for IV administration) and 0 648 494 (published April 19, 1995; illustrative formulation for oral administration).

The effective dose of the compound will typically be in the range of about 0.01 to about 50 mg/kgs, preferably about 0.1 to about 10 mg/kg of mammalian body weight, administered in single or multiple doses. Generally, the compound may be administered to patients in need of such treatment in a daily dose range of about 1 to about 2000 mg per patient.

The amount of compound which will be effective in the treatment or prevention of a particular disorder or condition will depend in part on the characteristics of the fusion proteins to be multimerized, the characteristics and location of the genetically engineered cells, and on the nature of the disorder or condition, which can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. The precise dosage level should be determined by the attending physician or other health care provider and will depend upon well known factors, including route of administration, and the age, body weight, sex and general health of the individual; the nature, severity and clinical stage of the disease; the use (or not) of concomitant therapies; and the nature and extent of genetic engineering of cells in the patient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers containing one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. The notice or package insert may contain instructions for use of an improved rapalog of this invention, consistent with the disclosure herein.

25

**1.4. Rapamycin (E and Z)-24-(O-isobutyloxime) (9, 10)**

Prepared in an analogous manner to Rapamycin (E and Z)-24-(O-methyloxime). The isomer mixture was separated by HPLC (15% H<sub>2</sub>O/MeCN through a Kromasil C-18 250 x 20 mm column, 12 mL/min) to provide 28 mg (65%) of the faster eluting Z isomer and 3.0 mg (7%) of the E isomer. Z isomer: high-resolution mass spectrum (FAB) m/z 1007.6146 [(M+Na)<sup>+</sup>, calcd for C<sub>55</sub>H<sub>88</sub>N<sub>2</sub>O<sub>13</sub>Na 1007.6184]. E isomer: high-resolution mass spectrum (FAB) m/z 1007.6157 [(M+Na)<sup>+</sup>, calcd for C<sub>55</sub>H<sub>88</sub>N<sub>2</sub>O<sub>13</sub>Na 1007.6184].

**1.5. Rapamycin (E and Z)-24-(O-benzyloxime) (11, 12)**

Prepared in an analogous manner to Rapamycin (E and Z)-24-(O-methyloxime). The isomer mixture was separated by HPLC (15% H<sub>2</sub>O/MeCN through a Kromasil C-18 250 x 20 mm column, 12 mL/min) to provide 19.6 mg (44%) of the faster eluting Z isomer and 6.1 mg (14%) of the E isomer. Z isomer: high-resolution mass spectrum (FAB) m/z 1041.6033 [(M+Na)<sup>+</sup>, calcd for C<sub>58</sub>H<sub>86</sub>N<sub>2</sub>O<sub>13</sub>Na 1041.6028]. E isomer: high-resolution mass spectrum (FAB) m/z 1041.5988 [(M+Na)<sup>+</sup>, calcd for C<sub>58</sub>H<sub>86</sub>N<sub>2</sub>O<sub>13</sub>Na 1041.6028].

**1.6. Rapamycin (E and Z)-24-(O-carboxymethyloxime) (13, 14)**

Prepared in an analogous manner to Rapamycin (E and Z)-24-(O-methyloxime). The isomer mixture was separated by HPLC (45% H<sub>2</sub>O/MeCN through a Kromasil C-18 250 x 20 mm column, 12 mL/min) to provide 4.6 mg (11%) of the faster eluting Z isomer and 1.0 mg (2%) of the E isomer. Z isomer: high-resolution mass spectrum (FAB) m/z 1009.5664 [(M+Na)<sup>+</sup>, calcd for C<sub>53</sub>H<sub>82</sub>N<sub>2</sub>O<sub>15</sub>Na 1009.5613]. E isomer: high-resolution mass spectrum (FAB) m/z 1009.5604 [(M+Na)<sup>+</sup>, calcd for C<sub>53</sub>H<sub>82</sub>N<sub>2</sub>O<sub>15</sub>Na 1009.5613].

**1.7. Rapamycin (E and Z)-24-(O-carboxamidomethyloxime) (15, 16)**

Prepared in an analogous manner to Rapamycin (E and Z)-24-(O-methyloxime). The isomer mixture was separated by HPLC (35% H<sub>2</sub>O/MeCN through a Kromasil C-18 250 x 20 mm column, 12 mL/min) to provide 6.2 mg (10%) of the faster eluting Z isomer and 1.4 mg (2%) of the E isomer. Z isomer: high-resolution mass spectrum (FAB) m/z 1008.5790 [(M+Na)<sup>+</sup>, calcd for C<sub>53</sub>H<sub>83</sub>N<sub>3</sub>O<sub>14</sub>Na 1008.5768]. E isomer: high-resolution mass spectrum (FAB) m/z 1008.5753 [(M+Na)<sup>+</sup>, calcd for C<sub>53</sub>H<sub>83</sub>N<sub>3</sub>O<sub>14</sub>Na 1008.5768].

**Example 2. Assay of binding of rapamycin C24 derivatives to FKBP**

Affinities of rapamycin C24 analogs for FKBP were determined using a competitive assay based on fluorescence polarization (FP). A fluorescein-labelled FK506 probe (AP1491) was synthesized, and the increase in the polarization of its fluorescence used as a direct readout of

organic layer was decanted, dried over anhydrous sodium sulfate containing a small amount of sodium sulfite, filtered, and concentrated. The residue was dissolved in anhydrous THF (2.8 mL), cooled to -78°C under nitrogen, and treated with a 0.5 M solution of lithium tris [(3-ethyl-3-pentyl)oxy]aluminum hydride in THF (282 µL). The resulting solution was stirred at -78°C for 1.75 h, and then quenched by addition of ether (6 mL) and saturated ammonium chloride solution (250 µL). The mixture was allowed to warm up to room temperature and treated with anhydrous sodium sulfate. Filtration and concentration under reduced pressure afforded a pale yellow oil (97 mg), which was purified by column chromatography (silica-gel, hexanes-EtOAc 3:1) to afford 1 as a colorless oil.

### 2.3 Intermediate 2

A solution of the above alcohol (300 mg, 290 µmol) in acetonitrile (10 mL) was treated with 2,6-lutidine (338 µL, 2.9 mmol) and N,N'-disuccinimidylcarbonate (371 mg, 1.45 mmol). The resulting suspension was stirred at room temperature for 14.5 h, and then concentrated under reduced pressure. The residue was chromatographed (silica-gel, hexanes-EtOAc 2:1 to 100% EtOAc gradient) to afford the mixed carbonate 2 as a pale yellow oil (127 mg).

### 2.4 Intermediate 3

A solution of the above carbonate (30 mg, 26 µmol) and triethylamine (36 µL, 260 µmol) in acetonitrile (1 mL) was treated with 4'-(aminomethyl)fluorescein (13.5 mg, 34 µmol). The resulting bright orange suspension was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was chromatographed (silica-gel, hexanes-EtOAc 1:1 to 100% EtOAc to EtOAc-MeOH 1:1 gradient) to give 3 (20.5 mg) as a bright yellow solid.

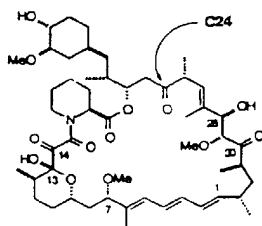
### 2.5 Compound 4

A solution of bis-silyl ether 3 (35 mg, 25 µmol) in acetonitrile (2 mL) was treated with 48% (w/w) HF in water (250 µL). The resulting mixture was stirred at room temperature for 5.5 h. It was then diluted with dichloromethane (10 mL) and washed with water (2x2 mL). The organic layer was decanted, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed (silica-gel, 100% EtOAc) to afford 4 (13 mg) as a bright yellow solid.

### 2.6 Determination of binding affinities (IC50s) of rapalogs using FP

Serial 10-fold dilutions of each analog were prepared in 100% ethanol in glass vials and stored on ice. All other manipulations were performed at room temperature. A stock of recombinant pure FKBP (purified by standard methods, see eg. Wiederrecht, G. *et al.* 1992. J. Biol. Chem.

Table 4



| cmpd       | C24 | isomer    | FKBPwt FP<br>binding assay<br>IC50 (nM) | fold loss in<br>affinity<br>(vs<br>rapamycin) |
|------------|-----|-----------|---|---|
| rapamycin  |     |           | 2.3                                     | (1)   |
| C14 desoxo |     |           | 63.3                                    | 27.5  |
| 17         |     | Z (major) | 618                                     | 269   |
| 18         |     | E (minor) | 59.1                                    | 25.7  |
| 5          |     | Z (major) | 1416                                    | 616   |
| 6          |     | E (minor) | 438                                     | 190   |
| 7          |     | Z (major) | 2960                                    | 1287  |
| 8          |     | E (minor) | 1664                                    | 723   |
| 9          |     | Z (major) | >30000                                  | >13043  |

$$y = M1 + (M4 - M1) / (1 + \exp(M2 * (M3 - x)))$$

where M3 is the IC50. For incomplete curves the IC50 was determined by interpolation. Rapamycin and C14-desoxo-rapamycin were included as controls in each case (C14-desoxo-rapamycin was prepared as described by Luengo, J.I. *et al.* 1994 Tet Lett. 35, 6469-6472).

5

## 2.7 Results of binding analysis of Rapamycin C24 oximes

Affinities are reported as IC50s and as fold loss in affinity (= IC50 / IC50 of rapamycin). (Comparative binding data of C24 rapalogs vs rapamycin and desoxo-rapamycin towards human FKBP12 are plotted in PCT/US86/09848.)

10

### Example 3. Synthesis of C7 rapalogs; Assay of binding of C7 rapalog-FKBP complexes to FRAP

A series of C7 rapalogs containing various C7 substituents selected from branched and unbranched alkoxy, arylalkoxy, -NHCO-Oalkyl, -NHSO<sub>2</sub>alkyl and substituted aryl and heteroaryl moieties was synthesized using chemistry generally as described in the literature except as noted (see *e.g.*, Luengo *et al.* 1995. Chemistry and Biology 2, 471-481, and the references cited in Table II for additional background). See also the table which follows.

15

3.1 Compounds 27, 28 - (R<sup>C7</sup>=Et) are synthesized as described in Luengo *et al.*, Chemistry & Biology July 1995, 2:471-481.

20

3.2 Compound 29 - (R<sup>C7</sup>=iPr) A solution of rapamycin (60 mg, 0.066 mmol) in 2-propanol (3 mL) at room temperature was treated with para-toluenesulfonic acid (75 mg, 0.394 mmol) and allowed to stir for 4 h. After this time the reaction was poured onto a biphasic solution of saturated aqueous NaHCO<sub>3</sub> (20 mL) and EtOAc (30 mL). The organic layer was washed with additional solution of saturated aqueous NaHCO<sub>3</sub> (2 x 20 mL) followed by a saturated aqueous solution of NaCl (2 x 10 mL) then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, evaporated. The resulting material was purified by HPLC on a Kromasil C-18 column (20 x 250 mm) at 55 C using 65% acetonitrile/water as eluant to afford AP1700 (25 mg). MS(FAB): (M+Na)<sup>+</sup> calcd: 964.5762, found: 964.5753.

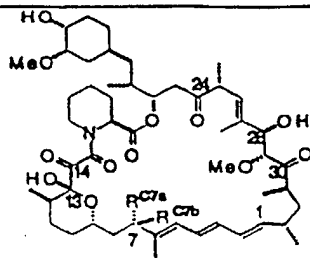
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30

3.3 Compound 30 - (R<sup>C7</sup>=benzyl) is synthesized as described in Chemistry & Biology July 1995, 2:471-481.

3.4 Compounds 31 32 - (R<sup>C7</sup>= -NH-CO-OMe) may be synthesized as described in Chemistry & Biology July 1995, 2:471-481.

35



| #                   | R <sup>C7a</sup>       | R <sup>C7b</sup> | #  | R <sup>C7a</sup>        | R <sup>C7b</sup>        |
|---------------------|------------------------|------------------|----|-------------------------|-------------------------|
| rapamycin           | -OMe                   | H                | 41 |                         | H                       |
| C14-deoxo rapamycin | -OMe                   | H                | 40 | **                      |                         |
| 27                  | -OEt                   | H                | 42 | -o,p-dimethoxyphenyl    | H                       |
| 28                  | H                      | -OEt             | 43 | H                       | -o,p-dimethoxyphenyl    |
| 29                  | -O-iPr                 | H                | 44 |                         | H                       |
| 30                  | -O-benzyl              | H                | 45 | H                       |                         |
| 32                  | -NH-(C=O)-OMe          | H                | 46 | -o,p-diethoxyphenyl     | H                       |
| 31                  | H                      | -NH-(C=O)-OMe    | 47 |                         | H                       |
| 33                  | -NH-SO <sub>2</sub> Me | H                | 48 |                         | H                       |
| 34                  |                        | H                | 49 | -2,4,6-trimethoxyphenyl | H                       |
| 35                  | H                      |                  | 50 | H                       | -2,4,6-trimethoxyphenyl |
| 36                  |                        | H                | 51 | -NH-(C=O)-OEt           | H                       |
| 37                  | H                      |                  | 52 | H                       | -NH-(C=O)-OEt           |
| 38                  |                        | H                |    |                         |                         |
| 39                  | H                      |                  |    |                         |                         |

silica gel (dichloromethane:hexane:EtOAc:MeOH, 200:50:42.5:7.5). The resulting material was purified by HPLC on a Rainin silica column (20 x 250 mm) using (dichloromethane:hexane:EtOAc:MeOH, 210:65:65:10) as eluant for AP20808 (20 mg). MS(ES+): (M+Na)<sup>+</sup> 1065.95.

5

**3.13 Compound 47** - (R<sup>C7</sup> = methylthiophene) A solution of rapamycin (105 mg, 0.115 mmol) and 3-methylthiophene (445 µL, 4.60 mmol) in dichloromethane (2.0 mL) at -40 °C was treated dropwise with trifluoroacetic acid (150 µL, 1.96 mmol) and allowed to stir for 3 h. After this time the reaction was poured onto a biphasic solution of saturated aqueous NaHCO<sub>3</sub> (15 mL)

10 and EtOAc (15 mL). The organic layer was washed with a saturated aqueous solution of NaCl (2 x 10 mL) then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, evaporated, and flash chromatographed on a silica gel (dichloromethane:hexane:EtOAc:MeOH, 200:50:42.5:7.5). The resulting material was purified by HPLC on a Rainin silica column (20 x 250 mm) using (dichloromethane:hexane:EtOAc:MeOH, 210:65:65:10) as eluant for AP20809 (60 mg). MS(ES+):  
15 (M+Na)<sup>+</sup> 1002.96.

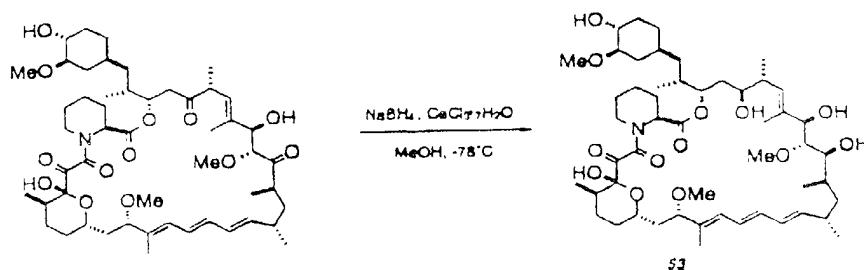
**3.14 Compound 48** - (R<sup>C7</sup> = N-methylpyrrole) A solution of rapamycin (51 mg, 0.056 mmol) and N-methylpyrrole (198 µL 2.23 mmol) in dichloromethane (2.0 mL) at 0 °C was treated with zinc chloride (76 mg, 0.557 mmol) and allowed to warm to rt overnight. After this time the reaction  
20 was poured onto a biphasic solution of saturated aqueous NaHCO<sub>3</sub> (15 mL) and EtOAc (15 mL). The organic layer was washed with a saturated aqueous solution of NaCl (2 x 10 mL) then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, evaporated, and flash chromatographed on a silica gel (dichloromethane:hexane:EtOAc:MeOH, 100:150:150:10). The resulting material was purified  
25 by HPLC on a Rainin Si column (20 x 250 mm) using (dichloromethane:hexane:EtOAc:MeOH, 210:65:65:10) as eluant for AP20810 (10 mg). MS(ES+): (M+NH<sub>4</sub>)<sup>+</sup> 981.05; MS(ES-): (M-H)<sup>-</sup> 961.69.

The C7 rapalogs were characterized by exact mass spec and NMR.

30 **3.15 Assay of FKBP binding affinity of C7 rapalogs**

The affinity of a variety of the C7 rapalogs for FKBP was assayed as described for C24 rapalogs above, using competitive FP. Rapamycin and C14-desoxo-rapamycin (prepared as described by Luengo *et al.* 1994. Tetrahedron Lett. 35, 6469-6472) were included as controls.

**Example 4 Preparation of Rapalogs modified at R<sup>C24</sup> and R<sup>C30</sup>; 24(S),30(S)-tetrahydrorapamycin (53)**

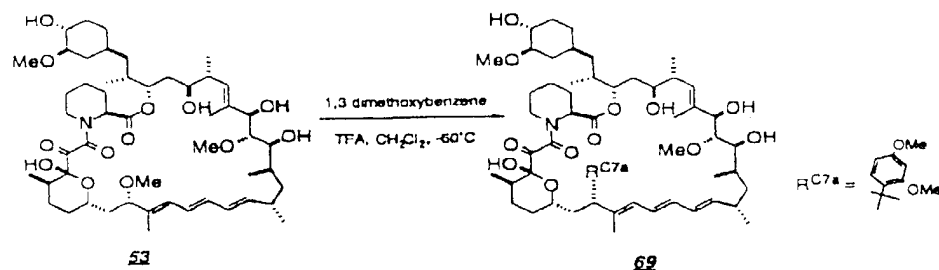


5  
Rapamycin (46 mg, 0.050 mmol) was dissolved in 2.0 mL of methanol, cooled to -78°C, and cerium  
(III) chloride heptahydrate (46 mg, 0.123 mmol) was added. The solution was stirred for 0.25  
h., then sodium borohydride (7.6 mg, 0.20 mmol) was added. After 0.5 h, the reaction mixture  
10 was partitioned between ethyl acetate (15 mL) and 5% aqueous hydrochloric acid (2 mL). The  
organic phase was washed with water (2 mL) and brine (1 mL), dried over anhydrous  
magnesium sulfate, filtered, and concentrated. Flash chromatography (silica gel, 15 : 75 : 50 :  
200 methanol : ethyl acetate : hexane : dichloromethane) yielded 35 mg (76%) of the desired  
product as a white foam. Mass spectral data: (ES+/ NaCl / NH3) m/z 942.21 (M+Na)+, 935.83  
15 (M+NH4)+; (ES-/ NaCl) m/z 963.04 (M+Cl)-, 917.34 (M-H)- lit. ref. Luengo, J.I.; Rozamus,  
L.W.; Holt, D.A. Tetrahedron Lett. 1994, 35, 6469-6472.

**Example 5 Preparation of Rapalogs modified at C24, C30 and C7**

24(S), 30(S)-tetrahydrorapamycin (53), prepared as in Example 4, may be modified at C7 using  
20 approaches illustrated in the prior C7 rapalog examples. For example:

**5.1 7(S)-(2',4'-dimethoxy)benzyl-7-demethoxy-24(S), 30(S)-tetrahydro-rapamycin**



25  
24(S), 30(S)-tetrahydro-rapamycin (20 mg, 0.022 mmol) was dissolved in dichloro-methane  
(1.0 mL). 1,3-dimethoxybenzene (0.20 mL, 1.5 mmol) was added, and the solution was cooled



to -60°C. Trifluoroacetic acid (0.030 mL, 0.39 mmol) was added, and the reaction mixture was stirred for 1 h at -60°C, then partitioned between ethyl acetate (10 mL) and saturated aqueous sodium bicarbonate (1 mL). The organic phase was washed with water (2 mL) and brine (1 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated. Flash chromatography (silica gel, 15 : 75 : 50 : 200 methanol : ethyl acetate : hexane : dichloromethane) yielded 8 mg (35%) of the desired product as a white solid. Mass spectral data: (ES+/ NaCl / NH3) m/z 1046.96 (M+Na)+, 1042.15 (M+NH4)+; (ES-/ NaCl) m/z 1069.09 (M+Cl)- lit. ref. Luengo, J.I.; Konialian-Beck, A.; Rozamus, L.W.; Holt D.A. J. Org. Chem. 1994, 59, 6512-6513.

By analogous means, one may produce 24(S), 30(S)-tetra-, two rapamycins bearing other C7 substituents as described elsewhere herein, e.g., containing alternatively substituted aryl groups, heteroaryl, -O-aliphatic groups, thioethers, or any of the other types of moieties designated previously for R<sup>C7a</sup> or R<sup>C7b</sup>. These compounds may be obtained by reduction at C24 and C30 of the appropriate C7 rapalog, or by transformation at C7 of the appropriate C24, C30-tetrahydro rapalog. Illustrative examples follow.

Rapalogs modified at C24, C30 and C7 may also be differ from rapamycin at the various positions discussed herein, e.g. with respect to one or more of R<sup>C13</sup>, R<sup>C43</sup>, R<sup>C28</sup>, R<sup>C29</sup>, R<sup>4</sup>, "a", etc. By way of example, starting with 13-F- rapamycin in place of rapamycin yields the 13-fluoro analogs of compounds 53-79.

5.2 Compounds 54, 55 - (R<sup>C7</sup>=Et) are synthesized as described in Example 4.1, but substituting Compounds 22 and 28, respectively, for rapamycin.

5.3 Compound 56 - (R<sup>C7</sup>=iPr) is synthesized as described in Example 4.1, but substituting Compound 29 for rapamycin.

5.4 Compound 57 - (R<sup>C7</sup>=benzyl) is synthesized as described in Example 4.1, but substituting Compound 30 for rapamycin.

5.5 Compounds 58, 59 - (R<sup>C7</sup>= -NH-CO-OMe) are synthesized as described in Example 4.1, but substituting Compounds 32 and 31, respectively, for rapamycin.

- 5.6 Compound 60 - ( $R^{C7}$  = -NH-SO<sub>2</sub>-Me) is synthesized as described in Example 4.1, but substituting Compound 33 for rapamycin
- 5.7 Compounds 61 and 62 - ( $R^{C7}$  = furanyl) are synthesized as described in Example 4.1, but  
5 substituting Compounds 34 and 35, respectively, for rapamycin.
- 5.8 Compounds 63, 64 - ( $R^{C7}$  = methylthiophene) are synthesized as described in Example 4.1, but substituting Compounds 36 and 37, respectively, for rapamycin.
- 10 5.9 Compounds 65, 66 - ( $R^{C7}$  = ethylthiophene) are synthesized as described in Example 4.1, but substituting Compounds 38 and 39, respectively, for rapamycin.
- 5.10 Compounds 67, 68 - ( $R^{C7}$  = tertbutyl thiophene) are synthesized as described in Example 4.1, but substituting Compounds 41 and 40, respectively, for rapamycin.
- 15 5.11 Compounds 69, 70 - ( $R^{C7}$  = o,p-dimethoxyphenyl) are synthesized as described in Example 4.1, but substituting Compounds 43 and 42, respectively, for rapamycin.
- 5.12 Compounds 71, 72 - ( $R^{C7}$  = indolyl) are synthesized as described in Example 4.1, but  
20 substituting Compounds 45 and 44, respectively, for rapamycin.
- 5.13 Compound 73 - ( $R^{C7}$  = o,p-diethoxyphenyl) is synthesized as described in Example 4.1, but substituting Compound 46 for rapamycin.
- 25 5.14 Compound 74 - ( $R^{C7}$  = methylthiophene) is synthesized as described in Example 4.1, but substituting Compound 47 for rapamycin.

| #  | R <sup>1</sup>         | R <sup>2</sup> | #  | R <sup>3</sup>                   | R <sup>4</sup>                   |
|----|------------------------|----------------|----|----------------------------------|----------------------------------|
| 53 | -OMe                   | H              | 67 |                                  | H                                |
| 54 | -OBz                   | H              | 68 | H                                |                                  |
| 55 | H                      | -OBz           | 69 | -o,p-(MeO) <sub>2</sub> phenyl   | H                                |
| 56 | -O-iPr                 | H              | 70 | H                                | -o,p-(MeO) <sub>2</sub> phenyl   |
| 57 | -O-benzyl              | H              | 71 |                                  | H                                |
| 58 | -NH-(C=O)-OMe          | H              | 72 | H                                |                                  |
| 59 | H                      | -NH-(C=O)-OMe  | 73 | -o,p-dichlorophenyl              | H                                |
| 60 | -NH-SO <sub>2</sub> Me | H              | 74 |                                  | H                                |
| 61 |                        | H              | 75 |                                  | H                                |
| 62 | H                      |                | 76 | -2,4,6-(MeO) <sub>3</sub> phenyl | H                                |
| 63 |                        | H              | 77 | H                                | -2,4,6-(MeO) <sub>3</sub> phenyl |
| 64 | H                      |                | 78 | -NH-(C=O)-OBz                    | H                                |
| 65 |                        | H              | 79 | H                                | -NH-(C=O)-OBz                    |
| 66 | H                      |                |    |                                  |                                  |

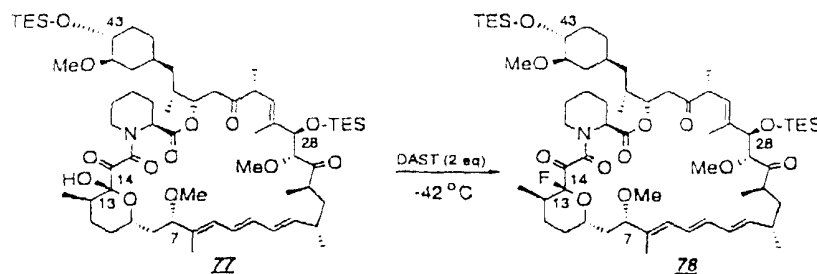
5.15 Compound **75** ( $R^C = \text{N-methylpyrrole}$ ) is synthesized as described in Example 4.1, but substituting Compound **48** for rapamycin.

- 5 5.16 Compound **75**, **76** ( $R^C = 2,4,6\text{-trimethoxyphenyl}$ ) is synthesized as described in Example 5.1, but substituting 1,3,5-trimethoxybenzene for 1,3-dimethoxybenzene.

#### Example 6. Preparation of fluoro-rapalogs

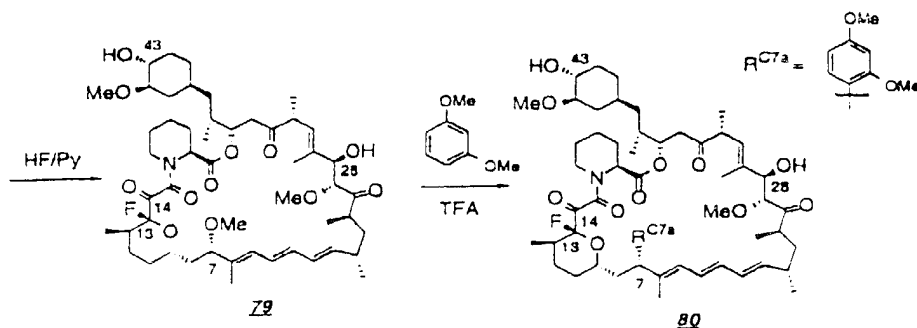
##### 10 6.1 C13-Fluoro-rapalogs

A new class of rapalogs, C13-Fluoro-rapalogs, may be prepared by the following route:



- In this example, the hydroxyl moieties at positions 28 and 43 are protected prior to treatment with DAST. We have used bis-triethylsilyl (as shown above) and bis-trisopropylsilyl protecting groups. Various alternative protecting groups may be used, based on user preference or convenience and in consideration of the reaction conditions of subsequent transformations prior to or concurrent with removal of protecting groups. The protected compound is then treated with the DAST reagent to introduce the 13-fluoro substituent. The DAST reaction may be conducted, e.g., at  $-42^\circ\text{C}$  as shown, or at  $0^\circ\text{C}$ .

- 13-Fluoro rapamycin may then be modified at position 7 as desired to produce the family of 13-fluoro C7-rapalogs bearing any of the variety of moieties designated previously for  $R^{C7a}$  or  $R^{C7b}$ . For instance, the 7-(o,p-dimethoxy)-13-fluoro-rapalogs (**96** and **97**) may be prepared (and separately recovered if desired) by transformation of **78** at C7 followed by removal of protecting groups, or, as shown below, by removal of protecting groups from **78** followed by transformation at C7.



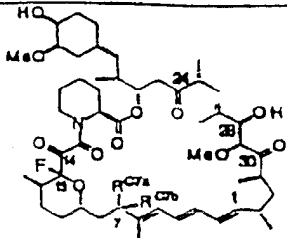
One may subject 13-F-rapamycin, instead of rapamycin, to various other chemical transformations such as are disclosed or referred to herein, including, for instance, fluorination at C28, reduction at C24 and C30, fluorination at C24 and C30, modification at C-43, etc., in addition to or as an alternative to modification at C7, in order to obtain the corresponding 13-F analog.











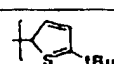

6.2 Compounds **81**, **82** - (R<sup>C7</sup>=Et) are synthesized as described in Example 3.1, but substituting 13-F-rapamycin (**79**) for rapamycin.

6.3 Compound **83** - (R<sup>C7</sup>=iPr) is synthesized as described in Example 3.2, but substituting 13-F-rapamycin (**79**) for rapamycin.

6.4 Compound **84** - (R<sup>C7</sup>=benzyl) is synthesized as described in Example 3.3, but substituting 13-F-rapamycin (**79**) for rapamycin.

6.5 Compounds **85**, **86** - (R<sup>C7</sup>=-NH-CO-OMe) are synthesized as described in Example 3.4, but substituting 13-F-rapamycin (**79**) for rapamycin.



| #  | R <sup>C1a</sup>  | R <sup>C1b</sup>  | #   | R <sup>C1a</sup>  | R <sup>C1b</sup>   |
|----|---|---|-----|---|--|
| 81 | -OEt  | H   | 96  | -o,p-(MeO) <sub>2</sub> phenyl  | H  |
| 82 | H   | -OEt  | 97  | H   | -o,p-(MeO) <sub>2</sub> phenyl   |
| 83 | -O-iPr  | H   | 98  |    | H  |
| 84 | -O-benzyl   | H   | 99  | H   |  |
| 85 | -NH-(C=O)-OMe   | H   | 100 | -o,p-diethoxyphenyl   | H  |
| 86 | H   | -NH-(C=O)-OMe   | 101 |    | H  |
| 87 | -NH-SO <sub>2</sub> Me  | H   | 102 |  | H  |
| 88 |  | H   | 103 | -2,4,6-(MeO) <sub>3</sub> phenyl  | H  |
| 89 | H   |  | 104 | H   | -2,4,6-(MeO) <sub>3</sub> phenyl   |
| 90 |  | H   | 105 | -NH-(C=O)-OEt   | H  |
| 91 | H   |  | 106 | H   | -NH-(C=O)-OEt  |
| 92 |  | H   |     |   |  |
| 93 | H   |  |     |   |  |
| 94 |  | H   |     |   |  |
| 95 | H   |  |     |   |  |

6.6 Compound 87 - ( $R^{C7}$  = -NH-SO<sub>2</sub>-Me) is synthesized as described in Example 3.5, but substituting 13-F-rapamycin (79) for rapamycin.

5 6.7 Compounds 88 and 89 - ( $R^{C7}$  = furanyl) are synthesized as described in Example 3.6, but substituting 13-F-rapamycin (79) for rapamycin.

6.8 Compounds 90, 91 - ( $R^{C7}$  = methylthiophene) are synthesized as described in Example 3.7, but substituting 13-F-rapamycin (79) for rapamycin.

10

6.9 Compounds 92, 93 - ( $R^{C7}$  = ethylthiophene) are synthesized as described in Example 3.8, but substituting 13-F-rapamycin (79) for rapamycin.

6.10 Compounds 68, 69 - ( $R^{C7}$  = tertbutyl thiophene) are synthesized as described in Example 3.9, but substituting 13-F-rapamycin (79) for rapamycin.

15

6.11 Compounds 94, 95 - ( $R^{C7}$  = o,p-dimethoxyphenyl) are synthesized as described in Example 3.10, but substituting 13-F-rapamycin (79) for rapamycin.

20 6.12 Compounds 96, 97 - ( $R^{C7}$  = indolyl) are synthesized as described in Example 3.11, but substituting 13-F-rapamycin (79) for rapamycin.

6.13 Compound 98 - ( $R^{C7}$  = o,p-diethoxyphenyl) is synthesized as described in Example 3.12, but substituting 13-F-rapamycin (79) for rapamycin.

25

6.14 Compound 99 - ( $R^{C7}$  = methylthiophene) is synthesized as described in Example 3.13, but substituting 13-F-rapamycin (79) for rapamycin.

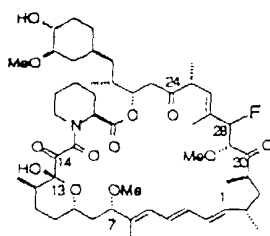
6.15 Compound 100 - ( $R^{C7}$  = N-methylpyrrole) is synthesized as described in Example 3.14, but substituting 13-F-rapamycin (79) for rapamycin.

30

#### 6.20 Preparation of 28-F-rapamycin (107)

To a solution of rapamycin (71 mg, 0.078 mmol) in methylene chloride (1 mL) at -78 °C was added DAST (21 mL, 0.156 mmol) and reaction was allowed to stir for 2h before MeOH was

added to quench the reaction. The reaction mixture was taken to room temperature and stirred for 30 min. It was poured onto a biphasic solution of saturated aqueous NaHCO<sub>3</sub> (20 mL) and EtOAc (30 mL). The organic layer was washed with additional solution of saturated aqueous NaHCO<sub>3</sub> (2 x 20 mL) followed by a saturated aqueous solution of NaCl (2 x 10 mL) then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, evaporated. The resulting material was flash chromatographed on a silica gel (hexane:EtOAc, 1:1 to 1:2). MS, Fluorine NMR indicated C28 fluorinated rapamycin. Stereoisomers can be separated by reverse phase chromatography (C18 column, MeOH:H<sub>2</sub>O, 80:20), and may be used in place of rapamycin for the synthesis of various F-28 rapalogs.



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6.21 Compound 108-(13-F, 28-F-rapamycin) is synthesized as described above for 28-F-rapamycin, but with twice the volume of DAST (41 mL) at a higher temperature (-40°C).

#### Example 7: Constructs encoding chimeric transcription factors

A Unless otherwise stated, all DNA manipulations described in this and other examples were performed using standard procedures (See e.g., F.M. Ausubel et al., Eds., Current Protocols in Molecular Biology (John Wiley & Sons, New York, 1994).

#### B. Plasmids

Constructs encoding fusions of human FKBP12 with the yeast GAL4 DNA binding domain, the HSV VP16 activation domain, human T cell CD3 zeta chain intracellular domain or the intracellular domain of human FAS are disclosed in PCT/US94/01617.

Additional DNA vectors for directing the expression of fusion proteins relevant to this invention were derived from the mammalian expression vector pCGNN (Attar, R.M. and Gilman, M.Z. 1992. MCB 12: 2432-2443). Inserts cloned as XbaI-BamHI fragments into pCGNN are transcribed under the control of the human CMV promoter and enhancer sequences (nucleotides -522 to +72 relative to the cap site), and are expressed with an



optional epitope tag (a 16 amino acid portion of the H. influenzae hemagglutinin gene that is recognized by the monoclonal antibody 12CA5) and, in the case of transcription factor domains, with an N-terminal nuclear localization sequence (NLS; from SV40 T antigen).

Except where stated, all fragments cloned into pCGNN were inserted as XbaI-BamHI fragments that included a SpeI site just upstream of the BamHI site. As XbaI and SpeI produce compatible ends, this allowed further XbaI-BamHI fragments to be inserted downstream of the initial insert and facilitated stepwise assembly of proteins comprising multiple components. A stop codon was interposed between the SpeI and BamHI sites. For initial constructs, the vector pCGNN-GAL4 was additionally used, in which codons 1-94 of the GAL4 DNA binding domain gene were cloned into the XbaI site of pCGNN such that a XbaI site is regenerated only at the 3' end of the fragment. Thus XbaI-BamHI fragments could be cloned into this vector to generate GAL4 fusions, and subsequently recovered.

*(a) Constructs encoding GAL4 DNA binding domain- FRAP fusions*

To obtain portions of the human FRAP gene, human thymus total RNA (Clontech #64028-1) was reverse transcribed using MMLV reverse transcriptase and random hexamer primer (Clontech 1st strand synthesis kit). This cDNA was used directly in a PCR reaction containing primers 1 and 2 and Pfu polymerase (Stratagene). The primers were designed to amplify the coding sequence for amino acids 2025-2113 inclusive of human FRAP: an 89 amino acid region essentially corresponding to the minimal 'FRB' domain identified by Chen et al. (Proc. Natl. Acad. Sci. USA (1995) 92, 4947-4951) as necessary and sufficient for FKBP-rapamycin binding (hereafter named FRB). The appropriately-sized band was purified, digested with XbaI and SpeI, and ligated into XbaI-SpeI digested pCGNN-GAL4. This construct was confirmed by restriction analysis (to verify the correct orientation) and DNA sequencing and designated pCGNN-GAL4-1FRB.

Constructs encoding FRB multimers were obtained by isolating the FRB XbaI-BamHI fragment, and then ligating it back into pCGNN-GAL4-1FRB digested with SpeI and BamHI to generate pCGNN-GAL4-2FRB, which was confirmed by restriction analysis. This procedure was repeated analogously on the new construct to yield pCGNN-GAL4-3FRB and pCGNN-GAL4-4FRB.

Vectors were also constructed that encode larger fragments of FRAP, encompassing the minimal FRB domain (amino acids 2025-2113) but extending beyond it. PCR primers were designed that amplify various regions of FRAP flanked by 5' XbaI and 3' SpeI sites as indicated below.

| Designation | amino acid | 5' primer | 3' primer |
|-------------|------------|-----------|-----------|
|-------------|------------|-----------|-----------|

|       |           |   |   |
|-------|-----------|---|---|
| FRAPa | 2012-2127 | 6 | 7 |
| FRAPb | 1995-2141 | 5 | 8 |
| FRAPc | 1945-2113 | 3 | 2 |
| FRAPd | 1995-2113 | 5 | 2 |
| FRAPe | 2012-2113 | 6 | 2 |
| FRAPf | 2025-2127 | 1 | 7 |
| FRAPg | 2025-2141 | 1 | 8 |
| FRAPh | 2025-2174 | 1 | 4 |
| FRAPi | 1945-2174 | 3 | 4 |

Initially, fragment FRAPi was amplified by RT-PCR as described above, digested with XbaI and SpeI, and ligated into XbaI-SpeI digested pCGNN-GAL4. This construct, pCGNN-GAL4-FRAPi, was analyzed by PCR to confirm insert orientation and verified by DNA sequencing. It was then used as a PCR substrate to amplify the other fragments using the primers listed. The new fragments were cloned as GAL4 fusions as described above to yield the constructs pCGNN-GAL4-FRAPa, pCGNN-GAL4-FRAPb etc, which were confirmed by DNA sequencing.

Vectors encoding concatenates of two of the larger FRAP fragments, FRAPd and FRAPe, were generated by analogous methods to those used earlier. XbaI-BamHI fragments encoding FRAPd and FRAPe were isolated from pCGNN-GAL4-FRAPd and pCGNN-GAL4-FRAPe and ligated back into the same vectors digested with SpeI and BamHI to generate pCGNN-GAL4-2FRAPd and pCGNN-GAL4-2FRAPe. This procedure was repeated analogously on the new constructs to yield pCGNN-GAL4-3FRAPd, pCGNN-GAL4-3FRAPe, pCGNN-GAL4-4FRAPd and pCGNN-GAL4-4FRAPe. All constructs were verified by restriction analysis.

*(b) Constructs encoding FRB-VP16 activation domain fusions*

To generate N-terminal fusions of FRB domain(s) with the activation domain of the Herpes Simplex Virus protein VP16, the XbaI-BamHI fragments encoding 1, 2, 3 and 4 copies of FRB were recovered from the GAL4 fusion vectors and ligated into XbaI-BamHI digested pCGNN to yield pCGNN-1FRB, pCGNN-2FRB etc. These vectors were then digested with SpeI and BamHI. An XbaI-BamHI fragment encoding amino acids 414-490 of VP16 was isolated from plasmid pCG-Gal4-VP16 (Das, G., Hinkley, C.S. and Herr, W. (1995) Nature 374, 657-660) and ligated into the SpeI-BamHI digested vectors to generate pCGNN-1FRB-

VP16, pCGNN-2FRB-VP16, etc. The constructs were verified by restriction analysis and/or DNA sequencing.

*(c) Constructs encoding ZFHD1 DNA binding domain-FRB fusions*

- 5 An expression vector for directing the expression of ZFHD1 coding sequence in mammalian cells was prepared as follows. Zif268 sequences were amplified from a cDNA clone by PCR using primers 5'Xba/Zif and 3'Zif+G. Oct1 homeodomain sequences were amplified from a cDNA clone by PCR using primers 5'Not Oct HD and Spe/Bam 3'Oct. The Zif268 PCR fragment was cut with XbaI and NotI. The Oct1 PCR fragment was cut with NotI and BamHI. Both fragments were ligated in a 3-way ligation between the XbaI and BamHI sites of pCGNN (Attar and Gilman, 1992) to make pCGNNZFHD1 in which the cDNA insert is under the transcriptional control of human CMV promoter and enhancer sequences and is linked to the nuclear localization sequence from SV40 T antigen. The plasmid pCGNN also contains a gene for ampicillin resistance which can serve as a selectable marker.
- 10 (Derivatives, pCGNNZFHD1-FKBPx1 and pCGNNZFHD1-FKBPx3, were prepared containing one or three tandem repeats of human FKBP12 ligated as an XbaI-BamHI fragment between the SpeI and BamHI sites of pCGNNZFHD1. A sample of pCGNNZFHD1-FKBPx3 has been deposited with the American Type Culture Collection under ATCC Accession No. 97399. Sequences of primers is shown in WO 96/41865.
- 20 To generate C-terminal fusions of FRB domain(s) with the chimeric DNA binding protein ZFHD1, the XbaI-BamHI fragments encoding 1, 2, 3 and 4 copies of FRB were recovered from the GAL4 fusion vectors and ligated into Spe-BamHI digested pCGNN-ZFHD1 to yield pCGNN-ZFHD1-1FRB, pCGNN-ZFHD1-2FRB etc. Constructs were verified by restriction analysis and/or DNA sequencing.
- 25 To examine the effect of introducing additional 'linker' polypeptide between ZFHD1 and a C-terminal FRB domain, FRAP fragments encoding extra sequence N-terminal to FRB were cloned as ZFHD1 fusions. XbaI-BamHI fragments encoding FRAPa, FRAPb, FRAPc, FRAPd and FRAPe were excised from the vectors pCGNN-GAL4-FRAPa, pCGNN-GAL4-FRAPb etc and ligated into SpeI-BamHI digested pCGNN-ZFHD1 to yield the vectors
- 30 pCGNN-ZFHD1-FRAPa, pCGNN-ZFHD1-FRAPb, etc. Vectors encoding fusions of ZFHD1 to 2, 3 and 4 C-terminal copies of FRAPe were also constructed by isolating XbaI-BamHI fragments encoding 2FRAPe, 3FRAPe and 4FRAPe from pCGNN-GAL4-2FRAPe, pCGNN-GAL4-3FRAPe and pCGNN-GAL4-4FRAPe and ligating them into SpeI-BamHI digested pCGNN-ZFHD1 to yield the vectors pCGNN-ZFHD1-2FRAPe, pCGNN-ZFHD1-3FRAPe
- 35 and pCGNN-ZFHD1-4FRAPe. All constructs were verified by restriction analysis.

Vectors were also constructed that encode N-terminal fusions of FRB domain(s) with ZFHD1. XbaI-BamHI fragments encoding 1, 2, 3 and 4 copies of FRAPe were isolated from pCGNN-GAL4-1FRAPe, pCGNN-GAL4-2FRAPe etc and ligated into XbaI-BamHI digested pCGNN to yield the plasmids pCGNN-1FRAPe, pCGNN-2FRAPe etc. These vectors were  
5 then digested with SpeI and BamHI, and an XbaI-BamHI fragment encoding ZFHD1 (isolated from pCGNN-ZFHD1) ligated in to yield the constructs pCGNN-1FRAPe-ZFHD1, pCGNN-2FRAPe-ZFHD1 etc, which were verified by restriction analysis.

*(d) Constructs encoding FRB-p65 activation domain fusions*

10 To generate fusions of FRB domain(s) with the activation domain of the human NF-kB p65 subunit (hereafter designated p65), two fragments were amplified by PCR from the plasmid pCG-p65. Primers 9 (p65/ 5' Xba) and 11 (p65 3' Spe/Bam) amplify the coding sequence for amino acids 450-550, and primers 10 (p65/361 Xba) and 11 amplify the coding sequence for amino acids 361-550, both flanked by 5' XbaI and 3' SpeI/BamHI sites. PCR  
15 products were digested with XbaI and BamHI and cloned into XbaI-BamHI digested pCGNN to yield pCGNN-p65(450-550) and pCGNN-p65(361-550). The constructs were verified by restriction analysis and DNA sequencing.

DNA sequences encoding the 100 amino acid P65 transcription activation sequence and the more extended p65 transcription activation domain (351-550) are shown in WO  
20 96/41865.

To generate N-terminal fusions of FRB domain(s) with portions of the p65 activation domain, plasmids pCGNN-1FRB, pCGNN-2FRB etc were digested with SpeI and BamHI. An XbaI-BamHI fragment encoding p65 (450-550) was isolated from pCGNN-p65(450-550) and ligated into the SpeI-BamHI digested vectors to yield the plasmids pCGNN-1FRB-  
25 p65(450-550), pCGNN-2FRB-p65(450-550) etc. The construct pCGNN-1FRB-p65(361-550) was made similarly using an XbaI-BamHI fragment isolated from pCGNN-p65(361-550). These constructs were verified by restriction analysis.

To examine the effect of introducing additional 'linker' polypeptide between the p65 activation domain and an N-terminal FRB domain, FRAP fragments encoding extra sequence  
30 C-terminal to FRB were cloned as p65 fusions. XbaI-BamHI fragments encoding FRAPa, FRAPb, FRAPf, FRAPg and FRAPh were excised from the vectors pCGNN-GAL4-FRAPa, pCGNN-GAL4-FRAPb etc and ligated into XbaI-BamHI digested pCGNN to yield the vectors pCGNN-FRAPa, pCGNN-FRAPb, etc. These plasmids were then digested with SpeI and BamHI, and a XbaI-BamHI fragment encoding p65 (amino acids 450-550) ligated in to  
35 yield the five vectors pCGNN-FRAPa-p65, pCGNN-FRAPb-p65, etc, which were verified by restriction analysis.

Vectors encoding fusions of p65 to 1 and 3 N-terminal copies of FRAPe were also prepared by digesting pCGNN-1FRAPe and pCGNN-3FRAPe with SpeI and BamHI. XbaI-BamHI fragments encoding p65(450-550) and p65(361-550) (isolated from pCGNN-p65(450-550) and pCGNN-p65(361-550)) were then ligated in to yield the vectors pCGNN-1FRAPe-p65(450-550), pCGNN-3FRAPe-p65(450-550), pCGNN-1FRAPe-p65(361-550) and pCGNN-3FRAPe-p65(361-550). All constructs were verified by restriction analysis.

Vectors were also constructed that encode C-terminal fusions of FRB domain(s) with portions of the p65 activation domain. Plasmids pCGNN-p65(450-550) and pCGNN-p65(361-550) were digested with SpeI and BamHI, and XbaI-BamHI fragments encoding 1 and 3 copies of FRAPe (isolated from pCGNN-GAL4-1FRAPe and pCGNN-GAL4-3FRAPe) and 1 copy of FRB (isolated from pCGNN-GAL4-1FRB) ligated in to yield the plasmids pCGNN-p65(450-550)-1FRAPe, pCGNN-p65(450-550)-3FRAPe, pCGNN-p65(361-550)-1FRAPe, pCGNN-p65(361-550)-3FRAPe, pCGNN-p65(450-550)-1FRB and pCGNN-p65(361-550)-1FRB. All constructs were verified by restriction analysis.

(e) *Further constructs*

Other constructs can be made analogously with the above procedures, but using alternative portions of the FRAP sequence or FRB domains containing modified peptide sequence. For example, primers 12 and 13 are used to amplify the entire coding region of FRAP. Primers 1 and 13, 6 and 13, and 5 and 13, are used to amplify three fragments encompassing the FRB domain and extending through to the C-terminal end of the protein (including the lipid kinase homology domain). These fragments differ by encoding different portions of the protein N-terminal to the FRB domain. In each case, RT-PCR is used as described above to amplify the regions from human thymus RNA, the PCR products are purified, digested with XbaI and SpeI, ligated into XbaI-SpeI digested pCGNN, and verified by restriction analysis and DNA sequencing.

(f) *Primer sequences*

```

1  5' GCATGTCTAGAGAGATGTGGCATGAAGGCCTGGAAG
2  5' GCATCACTAGTCTTTGAGATTCGTCGGAACACATG
3  5' GCACATTCTAGAAATTGATACCCCCAGACCCTTG
4  5' CGATCAACTAGTAAGTGTCAATTCCGGGGCCT
5  5' GCACTATCTAGACTGAAGAACATGTGTGAGCACAGC
6  5' GCACTATCTAGAGTGACGAGGAGCTGATCCGAGTG
7  5' CGATCAACTAGTGGAAACATATTGCAGCTCTAAGGA
8  5' CGATCAACTAGTTGGCACAGCCAATTCAAGGTCCCG

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9 5' ATGCTCTAGACTGGGGGCCTTGCTTGGCAAC  
 10 5' ATGCTCTAGAGATGAGTTTCCCACCATGGTG  
 11 5' GCATGGATCCGCTCAACTAGTGGAGCTGATCTGACTCAG  
 12 5' ATGCTCTAGACTTGAACCGGACCTGCCGCC  
 5 13 5' GCATCACTAGTCCAGAAAGGGCACCAGCCAATAT

Restriction sites are underlined (XbaI = TCTAGA, SpeI = ACGAGT, BamHI = GGATCC).

(g) DNA sequence of representative final construct: pCGNN-ZFHD1-1FRB

10 encoding a 12CA5 epitope-SV40 NLS-ZFHD1-FRB fusion is shown in WO 96/41865.

(h) Bicistronic constructs

The internal ribosome entry sequence (IRES) from the encephalomyocarditis virus was amplified by PCR from pWZL-Bleo. The resulting fragment, which was cloned into pBS-  
 15 SK+ (Stratagene), contains an XbaI site and a stop codon upstream of the IRES sequence and downstream of it, an NcoI site encompassing the ATG followed by SpeI and BamHI sites. To facilitate cloning, the sequence around the initiating ATG of pCGNN-ZFHD1-3FKBP was mutated to an NcoI site and the XbaI site was mutated to a NheI site using oligonucleotides shown in WO 96/41865. An NcoI-BamHI fragment containing ZFHD1-3FKBP was then  
 20 cloned downstream of pBS-IRES to create pBS-IRES-ZFHD1-3FKBP. The XbaI-BamHI fragment from this plasmid was next cloned into SpeI/BamHI-cut pCGNN-1FRB-p65(361-550) to create pCGNN-1FRB-p65(361-550)-IRES-ZFHD1-3FKBP.

C. Retroviral vectors for the expression of chimeric proteins

25 Retroviral vectors used to express transcription factor fusion proteins from stably integrated, low copy genes were derived from pSRaMSVtkNeo (Muller et al., MCB 11:1785-92, 1991) and pSRaMSV(XbaI) (Sawyers et al., J. Exp. Med. 181:307-313, 1995). Unique BamHI sites in both vectors were removed by digesting with BamHI, filling in with Klenow and religating to produce pSMTN2 and pSMTX2, respectively. pSMTN2 expresses the Neo  
 30 gene from an internal thymidine kinase promoter. A Zeocin gene (Invitrogen) is cloned as a NheI fragment into a unique XbaI site downstream of an internal thymidine kinase promoter in pSMTX2 to yield pSNTZ. This Zeocin fragment was generated by mutagenizing pZeo/SV (Invitrogen) using the following primers to introduce NheI sites flanking the zeocin coding sequence.

35 Primer 1 5'-GCCATGGTGGCTAGCCTATAGTGAG

Primer2 5'-GGCGGTGTTGGCTAGCGTCGGTCAG

pSMTN2 contains unique EcoRI and HindIII sites downstream of the LTR. To facilitate cloning of transcription factor fusion proteins synthesized as XbaI-BamHI fragments the following sequence was inserted between the EcoRI and HindIII sites to create pSMTN3:

12CA5 epitope

5' gaattccagaagcgcgt M A S S Y P Y D Y P D  
EcoRI

SV40 T NLS

Y A S L Q Q P S S P K K K R K  
TAT GCC AGC CTG GGA GGA CCT TCT AGT CCT AAG AAG AAG AGA AAG

V  
GTG TCT AGA TAT CGA GGA TCC CAA GCT T  
XbaI BamHI HindIII

The equivalent fragment is inserted into a unique EcoRI site of pSMTZ to create pSMTZ3 with the only difference being that the 3' HindIII site is replaced by an EcoRI site.

pSMTN3 and pSMTZ3 permit chimeric transcription factors to be cloned downstream of the 5' viral LTR as XbaI-BamHI fragments and allow selection for stable integrants by virtue of their ability to confer resistance to the antibiotics G418 or Zeocin respectively.

To generate the retroviral vector SMTN-ZFHD1-3FKBP, pCGNN-ZFHD1-3FKBP was first mutated to add an EcoRI site upstream of the first amino acid of the fusion protein. An EcoRI-BamHI(blunted) fragment was then cloned into EcoRI-HindIII(blunted) pSRaMSVtkNeo so that ZFHD1-3FKBP was expressed from the retroviral LTR.

#### Example 8: Rapamycin-dependent transcriptional activation

In preliminary experiments, three copies of FKBP fused either to a Gal4 DNA binding domain or a transcription activation domain activated both the stably integrated or transiently transfected reporter gene more strongly than corresponding fusion proteins containing only one or two FKBP domains. To evaluate this parameter with FRB fusion proteins, effector plasmids containing Gal4 DNA binding domain fused to one or more copies of an FRB domain were co-transfected with a plasmid encoding three FKBP domains and a p65 activation domain (3xFKBP-p65) by transient transfection. It was found that in this system, four copies of the FRB domain fused to the Gal4 DNA binding domain activated the

stably integrated reporter gene more strongly than other corresponding fusion proteins with fewer FRB domains.

**Method:** HT1080 B cells were grown in MEM supplemented with 10% Bovine Calf Serum.

- 5 Approximately 4x10<sup>5</sup> cells/well in a 6 well plate (Falcon) were transiently transfected by Lipofection procedure as recommended by the supplier (GIBCO, BRL). The DNA: Lipofectamine ratio used in this experiment correspond to 1:6. Cells in each well recieved 500 ng of pCGNN F3-p65, 1.9 ug of PUC 118 plasmid as carrier and 100 ng of one of the following plasmids: pCGNN Gal4-1FRB, pCGNN Gal4-2FRB, pCGNN Gal4-3FRB or
- 10 pCGNN Gal4-4FRB. Following transfection, 2 ml fresh media was added and supplemented with Rapamycin to the indicated concentration. After 24 hrs, 100 ul of the media was assayed for SEAP activity as described (Spencer et al, 1993).

- To test whether multiple FRB domains fused to a p65 activation domain results in
- 15 increased transcriptional activation of the reporter gene, we co-transfected HT1080 B cells with plasmids expressing Gal4-3xFKBP and 1, 2, 3 or 4 copies of FRB fused to p65 activation domain. Surprisingly, unlike the DNA binding domain-FRB fusions, a single copy of FRB fused to p65 activation domain activated the reporter gene significantly more strongly than
- 20 corresponding fusion proteins containing 2 or more copies of FRB.

- Method:** HT1080 B cells were grown in MEM supplemented with 10% Bovine Calf Serum. Approximately 4x10<sup>5</sup> cells/well in a 6 well plate were transiently transfected by Lipofection procedure as recommended by GIBCO, BRL. The DNA: Lipofectamine ratio used
- 25 correspond to 1:6. Cells in each well recieved 1.9 ug of PUC 118 plasmid as carrier , 100 ng of pCGNNGal4F3 and 500 ng one of the following plasmids :pCGNN1, 2, 3 or 4 FRB-p65. Following transfection, 2 ml fresh media was added and supplemented with Rapamycin to the indicated concentration. After 24 hrs, 100 ul of the media was assayed for SEAP activity as described (Spencer et al. 1993).

- 30 Similar experiments were also conducted using another stable cell line (HT1080 B14) containing the 5xGal4-IL2-SEAP reporter gene and DNA sequences encoding a fusion protein containing a Gal4 DNA binding domain and 3 copies of FKBP stably integrated. These cells were transiently transfected with effector plasmids expressing p65 activation domain fused to 1 or more copies of an FRB domain. Similar to our observations with HT1080 B cells, in
- 35 these experiments effector plasmids expressing a single copy of FRB-p65 activation domain



fusion protein activated the reporter gene more strongly than others with 2 or more copies of FRB.

#### Example 9

5

#### A. Rapamycin-dependent transcriptional activation in transiently transfected cells: ZFHD1 and p65 fusions

Human fibrosarcoma cells transiently transfected with a SEAP target gene and plasmids encoding representative ZFHD-FKBP- and FRB-p65-containing fusion proteins exhibited rapamycin-dependent and dose-responsive secretion of SEAP into the cell culture medium. See Fig. 4A. SEAP production was not detected in cells in which one or both of the transcription factor fusion plasmids was omitted, nor was it detected in the absence of added rapamycin (Figure 4B). When all components were present, however, SEAP secretion was detectable at rapamycin concentrations as low as 0.5 nM (Figure 4A). Peak SEAP secretion was observed at 5 nM. Similar results have been obtained when the same transcription factors were used to drive rapamycin-dependent activation of an hGH reporter gene or a stably integrated version of the SEAP reporter gene made by infection with a retroviral vector. It is difficult to determine the fold activation in response to rapamycin since levels of SEAP secretion in the absence of drug are undetectable, but it is clear that in this system there is at least a 1000-fold enhancement over background levels in the absence of rapamycin. Thus, this system exhibits undetectable background activity and high dynamic range.

Several different configurations for transcription factor fusion proteins were explored (See WO 96/41865, Fig. 5). When FKBP domains were fused to ZFHD1 and FRBs to p65, optimal levels of rapamycin-induced activation occurred when there were multiple FKBP domains fused to ZFHD1 and fewer FRBs fused to p65. The preference for multiple drug-binding domains on the DNA-binding protein may reflect the capacity of these proteins to recruit multiple activation domains and therefore to elicit higher levels of promoter activity. The presence of only 1 drug-binding domain on the activation domain should allow each FKBP on ZFHD to recruit one p65. Any increase in the number of FRBs on p65 would increase the chance that fewer activation domains would be recruited to ZFHD, each one linked by multiple FRB-FKBP interactions.

35    **Methods:**

HT1080 cells (ATCC CCL-121), derived from a human fibrosarcoma, were grown in MEM supplemented with non-essential amino acids and 10% Fetal Bovine Serum. Cells plated in 24-well dishes (Falcon,  $6 \times 10^4$  cells/well) were transfected using Lipofectamine under conditions recommended by the manufacturer (GIBCO/BRL). A total of 300 ng of the following DNA was transfected into each well: 100 ng ZFHDx12-CMV-SEAP reporter gene, 2.5 ng pCGNN-ZFHD1-3FKBP or other DNA binding domain fusion, 5 ng pCGNN-1FRB-p65(361-550) or other activation domain fusion and 192.5 ng pUC118. In cases where the DNA binding domain or activation domain were omitted an equivalent amount of empty pCGNN expression vector was substituted. Following lipofection (for 5 hours) 500  $\mu$ l medium containing the indicated amounts of rapamycin was added to each well. After 24 hours, medium was removed and assayed for SEAP activity as described (Spencer et al, Science 252:1019-24, 1993) using a Luminescence Spectrometer (Perkin Elmer) at 350 nm excitation and 450 nm emission. Background SEAP activity, measured from mock-transfected cells, was subtracted from each value.

To prepare transiently transfected HT1080 cells for injection into mice (See below), cells in 100 mm dishes ( $2 \times 10^6$  cells/dish) were transfected by calcium phosphate precipitation for 16 hours (Gatz, C., Kaiser, A. & Wendenburg, R., 1991, Mol. Gen. Genet. 227, 229-237) with the following DNAs: 10 mg of ZHWTx12-CMV-hGH, 1 mg pCGNN-ZFHD1-3FKBP, 2 mg pCGNN-1FRB-p65(361-550) and 7 mg pUC118. Transfected cells were rinsed 2 times with phosphate buffered saline (PBS) and given fresh medium for 5 hours. To harvest for injection, cells were removed from the dish in Hepes Buffered Saline Solution containing 10 mM EDTA, washed with PBS/0.1% BSA/0.1% glucose and resuspended in the same at a concentration of  $2 \times 10^7$  cells/ml.

**Plasmids:** Construction of the transcription factor fusion plasmids is described above.

#### pZHWTx12-CMV-SEAP

This reporter gene, containing 12 tandem copies of a ZFHD1 binding site (Pomerantz et al., 1995) and a basal promoter from the immediate early gene of human cytomegalovirus (Boshart et al., 1985) driving expression of a gene encoding secreted alkaline phosphatase (SEAP), was prepared by replacing the NheI-HindIII fragment of pSEAP Promoter (Clontech) with an NheI-XbaI fragment containing 12 ZFHD binding sites shown in WO 96/41865 and an XbaI-HindIII fragment containing a minimal CMV promoter (-54 to +45), also shown in WO 96/41865.

pZHWTx12-CMV-hGH

Activation of this reporter gene leads to the production of hGH. It was constructed by replacing the HindIII-BamHI (blunted) fragment of pZHWTx12-CMV-SEAP (containing the SEAP coding sequence) with a HindIII (blunted) -EcoRI fragment from p0GH (containing an hGH genomic clone; Selden et al., MCB 6:3171-3179, 1986; the BamHI and EcoRI sites were blunted together).

#### pZHWTx12-IL2-SEAP

This reporter gene is identical to pZHWTx12-CMV-SEAP except the XbaI-HindIII fragment containing the minimal CMV promoter was replaced with the following XbaI-HindIII fragment containing a minimal IL2 gene promoter (-72 to +45 with respect to the start site; Sieberlist et al., MCB 6:3042-3049, 1986) (see WO 96/41865)

#### pLH

To facilitate the stable integration of a single, or few, copies of reporter gene the following retroviral vector was constructed. pLH (LTR-hph), which contains the hygromycin B resistance gene driven by the Moloney murine leukemia virus LTR and a unique internal ClaI site, was constructed as follows: The hph gene was cloned as a HindIII-ClaI fragment from pBabe Hygro (Morganstern and Land, NAR 18:3587-96, 1990) into BamHI-ClaI cut pBabe Bleo (resulting in the loss of the bleo gene; the BamHI and HindIII sites were blunted together).

#### pLH-ZHWTx12-IL2-SEAP

To clone a copy of the reporter gene containing 12 tandem copies of the ZFHD1 binding site and a basal promoter from the IL2 gene driving expression of the SEAP gene into the pLH retroviral vector, the MluI-ClaI fragment from pZHWTx12-IL2-SEAP (with ClaI linkers added) was cloned into the ClaI site of pLH. It was oriented such that the directions of transcription from the viral LTR and the internal ZFHD-IL2 promoters were the same.

#### pLH-G5-IL2-SEAP

To construct a retroviral vector containing 5 Gal4 sites embedded in a minimal IL2 promoter driving expression of the SEAP gene, a ClaI-BstBI fragment consisting of the following was inserted into the ClaI site of pLH such that the directions of transcription from the viral LTR and the internal Gal4-IL2 promoters were the same: A ClaI-HindIII fragment containing 5 Gal4 sites and regions -324 to -294 and -72 to +45 of the IL2 gene (shown

in WO 96/41865) and a HindIII-BstBI fragment containing the SEAP gene coding sequence (Berger et al., Gene 66:1-10, 1988) mutagenized to add a BstBI site immediately after the stop codon (shown in WO 96/41865).

5    **B. Rapamycin-dependent transcriptional activation in stably transfected cells**

The following experiments confirmed that this system exhibits similar properties in stably transfected cells. We generated stable cell lines by sequential transfection of a SEAP target gene and expression vectors for ZFHD1-3FKBP and 1FRB-p65, respectively. A pool of  
10 several dozen stable clones resulting from the final transfection exhibited rapamycin-dependent SEAP production. From this pool, we characterized several individual clones, many of which produced high levels of SEAP in response to rapamycin. Results from one such clone are shown in Fig. 4C of WO 96/41865. This clone produced SEAP at levels approximately forty times higher than the pool and significantly higher than transiently  
15 transfected cells. In an attempt to rigorously quantitate background SEAP production and induction ratio in this clone, we performed a second set of assays in which the length of the SEAP assay was increased by a factor of approximately 50 to detect any SEAP activity in untreated cells. Under these conditions, mock transfected cells produced 47 arbitrary fluorescence units, while the transfected clone produced 54 units in the absence of rapamycin  
20 and over 90,000 units at 100 nM rapamycin. Thus, in this stable cell line, background gene expression was negligible and the induction ratio (7 units to 90,000 units) was greater than four orders of magnitude.

To simplify the task of stable transfection, we used a bicistronic expression vector that directs the production of both ZFHD1-3FKBP and 1FRB-p65 through the use of an internal  
25 ribosome entry sequence (IRES). This expression plasmid was cotransfected, together with a zeocin-resistance marker plasmid, into a cell line carrying a retrovirally-transduced SEAP reporter gene, and a pool of approximately fifty drug-resistant clones was selected and expanded. This pool of clones also exhibited rapamycin-dependent SEAP production with no detectable background and a very similar dose-response curve to that observed in transiently  
30 transfected cells. This pool would be expected to contain individual clones with performance similar to the clone studied in Fig. 4C of WO 96/41865. Thus, rapamycin-responsive gene expression can be readily obtained in both transiently and stably transfected cells. In both cases, regulation is characterized by very low background and high induction ratios.

35    **Stable cell lines.** Helper-free retroviruses containing the reporter gene or DNA binding domain fusion were generated by transient co-transfection of 293T cells (Pear, W.S., Nolan,

G.P., Scott, M.L. & Baltimore D., 1993, Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA 90, 8392-8396) with a Psi(-) amphotropic packaging vector and the retroviral vectors pLH-ZHWTx12-IL2-SEAP or SMTN-ZFHD1-3FKBP, respectively. To generate a clonal cell line containing the reporter gene stably integrated, HT1080 cells infected with retroviral stock were diluted and selected in the presence of 300 mg/ml Hygromycin B. Individual clones from this and other cell lines described below were screened by transient transfection of the missing components followed by the addition of rapamycin as described above. All 12 clones analyzed were inducible and had little or no basal activity. The most responsive clone, HT1080L, was selected for further study.

HT20-6 cells, which contain the pLH-ZHWTx12-IL2-SEAP reporter gene, ZFHD1-3FKBP DNA binding domain and 1FRB-p65(361-550) activation domain stably integrated, were generated by first infecting HT1080L cells with SMTN-ZFHD1-3FKBP-packaged retrovirus and selecting in medium containing 500 mg/ml G418. A strongly responsive clone, HT1080L3, was then transfected with linearized pCGNN-1FRB-p65(361-550) and pZeoSV (Invitrogen) and selected in medium containing 250 mg/ml Zeocin. Individual clones were first tested for the presence of 1FRB-p65(361-550) by western. Eight positive clones were analyzed by addition of rapamycin. All eight had low basal activity and in six of them, gene expression was induced by at least two orders of magnitude. The clone that gave the strongest response, HT20-6, was selected for further analysis.

HT23 cells were generated by co-transfecting HT1080L cells with linearized pCGNN-1FRB-p65(361-550)-IRES-ZFHD1-3FKBP and pZeoSV and selecting in medium containing 250 mg/ml Zeocin. Approximately 50 clones were pooled for analysis.

For analysis, cells were plated in 96-well dishes ( $1.5 \times 10^4$  cells/well) and 200  $\mu$ l medium containing the indicated amounts of rapamycin (or vehicle) was added to each well. After 18 hours, medium was removed and assayed for SEAP activity. In some cases, medium was diluted before analysis and relative SEAP units obtained multiplied by the fold-dilution. Background SEAP activity, measured from untransfected HT1080 cells, was subtracted from each value.

#### Example 10: Rapamycin-dependent Production of hGH in Mice

**In Vivo Methods: Animals, husbandry, and general procedures.** Male nu/nu mice were obtained from Charles River Laboratories (Wilmington, MA) and allowed to acclimate for five days prior to experimentation. They were housed under sterile conditions, were allowed free access to sterile food and sterile water throughout the entire experiment, and

were handled with sterile techniques throughout. No immunocompromised animal demonstrated outward infection or appeared ill as a result of housing, husbandry techniques, or experimental techniques.

To transplant transiently transfected cells into mice,  $2 \times 10^6$  transfected HT1080 cells, were suspended in 100 ml PBS/0.1% BSA/0.1% glucose buffer, and administered into four intramuscular sites (approximately 25 ml per site) on the haunches and flanks of the animals. Control mice received equivalent volume injections of buffer alone.

Rapamycin was formulated for in vivo administration by dissolution in equal parts of N,N-dimethylacetamide and a 9:1 (v:v) mixture of polyethylene glycol (average molecular weight of 400) and polyoxyethylene sorbitan monooleate. Concentrations of rapamycin, in the completed formulation, were sufficient to allow for in vivo administration of the appropriate dose in a 2.0 ml/kg injection volume. The accuracy of the dosing solutions was confirmed by HPLC analysis prior to intravenous administration into the tail veins. Some control mice, bearing no transfected HT1080 cells, received 10.0 mg/kg rapamycin. In addition, other control mice, bearing transfected cells, received only the rapamycin vehicle.

Blood was collected by either anesthetizing or sacrificing mice via CO<sub>2</sub> inhalation. Anesthetized mice were used to collect 100 ml of blood by cardiac puncture. The mice were revived and allowed to recover for subsequent blood collections. Sacrificed mice were immediately exsanguinated. Blood samples were allowed to clot for 24 hours, at 4°C, and sera were collected following centrifugation at 1000 x g for 15 minutes. Serum hGH was measured by the Boehringer Mannheim non-isotopic sandwich ELISA (Cat No. 1 585 878). The assay had a lower detection limit of 0.0125 ng/ml and a dynamic range that extended to 0.4 ng/ml. Recommended assay instructions were followed. Absorbance was read at 405 nm with a 490 nm reference wavelength on a Molecular Devices microtiter plate reader. The antibody reagents in the ELISA demonstrate no cross reactivity with endogenous, murine hGH in diluent sera or native samples.

**hGH expression In Vivo.** For the assessment of dose-dependent rapamycin-induced stimulation of hGH expression, rapamycin was administered to mice approximately one hour following injection of HT1080 cells. Rapamycin doses were either 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, or 10.0 mg/kg. Seventeen hours following rapamycin administration, the mice were sacrificed for blood collection.

To address the time course of in vivo hGH expression, mice received 10.0 mg/kg of rapamycin one hour following injection of the cells. Mice were sacrificed at 4, 8, 17, 24, and 42 hours following rapamycin administration.

The ability of rapamycin to induce sustained expression of hGH from transplanted HT1080 cells was tested by repeatedly administering rapamycin. Mice were administered transfected HT1080 cells as described above. Approximately one hour following injection of the cells, mice received the first of five intravenous 10.0 mg/kg doses of rapamycin. The four remaining doses were given under anesthesia, immediately subsequent to blood collection, at 16, 32, 48, and 64 hours. Additional blood collections were also performed at 72, 80, 88, and 96 hours following the first rapamycin dose. Control mice were administered cells, but received only vehicle at the various times of administration of rapamycin. Experimental animals and their control counterparts were each assigned to one of two groups. Each of the two experimental groups and two control groups received identical drug or vehicle treatments, respectively. The groups differed in that blood collection times were alternated between the two groups to reduce the frequency of blood collection for each animal.

#### Results

Rapamycin elicited dose-responsive production of hGH in these animals (Fig. 6 of WO 96/41865). hGH concentrations in the rapamycin-treated animals compared favorably with normal circulating levels in humans (0.2–0.3 ng/ml). No plateau in hGH production was observed in these experiments, suggesting that the maximal capacity of the transfected cells for hGH production was not reached. Control animals—those that received transfected cells but no rapamycin and those that received rapamycin but no cells—exhibited no detectable serum hGH. Thus, the production of hGH in these animals was absolutely dependent upon the presence of both engineered cells and rapamycin.

The presence of significant levels of hGH in the serum 17 hours after rapamycin administration was noteworthy, because hGH is cleared from the circulation with a half-life of less than four minutes in these animals. This observation suggested that the engineered cells continued to secrete hGH for many hours following rapamycin treatment. To examine the kinetics of rapamycin control of hGH production, we treated animals with a single dose of rapamycin and then measured hGH levels at different times thereafter. Serum hGH was observed within four hours of rapamycin treatment, peaked at eight hours (at over one hundred times the sensitivity limit of the hGH ELISA), and remained detectable 42 hours after treatment. hGH concentration decayed from its peak with a half-life of approximately 11 hours. This half-life is several hundredfold longer than the half-life of hGH itself and approximately twice the half-life of rapamycin (4.6 hr) in these animals. The slower decay of serum hGH relative to rapamycin could reflect the presence of higher tissue concentrations of rapamycin in the vicinity of the implanted cells. Alternatively,

persistence of hGH production from the engineered cells may be enhanced by the stability of hGH mRNA.

Interestingly, administration of a second dose of rapamycin to these animals at 42 hr resulted in a second peak of serum hGH, which decayed with similar kinetics indicating that the engineered cells retained the ability to respond to rapamycin for at least two days. Therefore, to ascertain the ability of this system to elevate and maintain circulating hGH concentrations, we performed an experiment in which animals received multiple doses of rapamycin at 16-hour intervals. This interval corresponds to the time required for hGH levels to peak and then decline approximately half-way. According to this regimen, rapamycin concentration is predicted to approach a steady-state trough concentration of 1.7  $\mu\text{g/ml}$  after two doses (shown as dotted line in Fig. 8 of WO 96/41865). hGH levels should also approach a steady state trough concentration following the second dose. Treated animals indeed held relatively stable levels of circulating hGH in response to repeated doses of rapamycin. After the final dose, hGH levels remained constant for 16 hours and then declined with a similar half-life as rapamycin (6.8 hours for hGH versus 4.6 hours for rapamycin). These data suggest that upon multiple dosing, circulating rapamycin imparts tight control over the secretion of hGH from transfected cells *in vivo*. In particular, it is apparent that protein production is rapidly terminated upon withdrawal of drug.

## Discussion

These experiments demonstrate the feasibility of controlling the production of a secreted therapeutic protein from genetically engineered cells using a small-molecule drug. This system has many of the features required for use in human gene and cell therapy. It is characterized by very low background activity and high induction ratio. It functions independently of host physiology or any cell-type-specific factors. It is composed completely of human proteins. The controlling drug is well behaved *in vivo* and orally bioavailable.

With a system of this general design, it should be possible to provide stable and precisely titrated doses of secreted therapeutic proteins from engineered cells *in vivo*. Intermittent and pulsatile dosing should also be feasible. A considerable advantage of protein delivery from engineered cells under small-molecule control is that the rate of protein production at any given time is a function of the circulating concentration of the small-molecule drug. Therefore, the apparent pharmacokinetics of a therapeutic protein such as hGH can be dramatically altered. In our experiments, for example, the kinetics of circulating hGH delivered from engineered cells following a single administration of rapamycin are markedly different from those observed following a single administration of



recombinant protein. hGH administered to mice intravenously is cleared with a half-time of a few minutes, whereas hGH levels from engineered cells induced with rapamycin decayed with a half-time of approximately eleven hours. Even in humans, where the half-time for hGH clearance is approximately twenty minutes, injections must be given every other day, and serum hGH levels fluctuate dramatically. It is likely that protein delivery from engineered cells under precise pharmacologic control will lead to more effective therapy, particularly for proteins with poor pharmacokinetics or low therapeutic index.

The use of a small-molecule drug to link a DNA-binding domain and activation domain is an effective strategy for regulating gene expression in vivo. One especially attractive feature is that the system is entirely modular, allowing each component to be optimized and engineered independently. In contrast to bacterial repressors, which rely on relatively subtle allosteric intramolecular interactions to control DNA-binding activity, the dimerization strategy can be adapted to virtually any DNA-binding and activation domain. We have used here a DNA-binding domain of defined structure which readily supports rational engineering of DNA-binding affinity and new recognition specificities. Similarly, activation domains can be engineered for maximal potency and other suitable properties. Indeed, the engineered transcription factors used in these experiments elicit very high levels of gene expression relative to conventional promoter/enhancer systems, and further enhancements in either domain can be readily incorporated. The ability to introduce engineered transcription factors dedicated to the transcription of a single target gene provides opportunities to achieve lower backgrounds and substantially higher levels of gene expression in vivo than conventional expression vectors.

We have also chosen to construct our regulated transcription factors from human proteins to minimize the potential for recognition by the immune system. It has been reported that autologous T cells expressing a fusion protein composed of bacterial hygromycin phosphotransferase and herpes virus thymidine kinase were effectively recognized and eliminated by host cytotoxic T cells, even in AIDS patients with debilitated immune systems (Riddell, S.R., et al. T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nature Med.* 2, 216-223 (1996)). This observation suggests that the risk of immune recognition of heterologous proteins in engineered cells is a real one and that, therefore, the use of human proteins for performing regulatory functions in human cells is prudent. Although each individual component of our transcription factor fusion proteins is human in sequence, each protein contains junction peptides which could potentially be recognized as foreign. These junctions may be designed or selected, however, to minimize their presentation to the immune system, as discussed previously.

The principal limitation of rapamycin-based systems is the native biological activity of rapamycin, which, through inhibition of FRAP activity blocks cell-cycle progression leading to immunosuppression *in vivo*. However, the ability to introduce substituents or otherwise modify the structure of rapamycin to substantially reduce or abolish binding to FKBP and/or FRAP provides access to rapalogs devoid of undue immunosuppressive activity. Use of such rapalogs, especially improved rapalogs of this invention, together with correspondingly engineered FKBP and/or FRB domains should prove widely useful for the regulation of engineered protein production as well as the regulation of a wide variety of other biological processes in experimental animals and human gene therapy.

#### Example 11: FP Assay for rapalog binding to FKBP

Affinities of rapalogs for FKBP proteins may be determined using a competitive assay based on fluorescence polarization (FP). A fluorescein-labelled FK506 probe (AP1491) was synthesized as described in WO 96/41865 (See Example 6 therein), and the increase in the polarization of its fluorescence used as a direct readout of % bound probe in an equilibrium binding experiment containing sub-saturating FKBP and variable amounts of rapamycin analog as competitor.

#### Determination of binding affinities (IC<sub>50</sub>s) of rapalogs using FP

Serial 10-fold dilutions of each analog are prepared in 100% ethanol in glass vials and stored on ice. All other manipulations are performed at room temperature. A stock of recombinant pure FKBP (purified by standard methods, see eg. Wiederrecht, G. et al. 1992. J. Biol. Chem. 267, 21753-21760) is diluted to 11.25 nM in 50 mM potassium phosphate pH 7.8/150 mM NaCl/ 100µg/ml bovine gamma globulin ("FP buffer": prepared using only low-fluorescence reagents from Panvera) and 98 µl aliquots transferred to wells of a Dynatech micro-fluor black 96-well fluorescence plate. 2.0 µl samples of the rapalogs are then transferred in duplicate to the wells with mixing. Finally, a probe solution is prepared containing 10 nM AP1491 in 0.1% ethanol/FP buffer, and 100 µl added to each well with mixing. Duplicate control wells contain ethanol instead of rapalog (for 100% probe binding) or ethanol instead of rapalog and FP buffer instead of FKBP (0% binding).

The plates are stored covered in the dark for approximately 30 min to permit equilibration and then the fluorescence polarization of the sample in each well is read on a Jolley FPM-2 FP plate reader (Jolley Consulting and Research, Inc., Grayslake, IL) in accordance with the manufacturer's recommendations. The mean polarization (mP units) for each competitor concentration is usually converted to % total binding by reference to the control values and plotted (y) vs. log molar final concentration of competitor (x). Non-linear

least square analysis was used to fit the curve and extract the IC50 using the following equation:

$$y = M1 + (M4 - M1) / (1 + \exp(M2 * (M3 - x)))$$

- 5 where M3 is the IC50. For incomplete curves the IC50 is determined by interpolation. Rapamycin and C14-desoxo-rapamycin may be included as controls in each case ( C14-desoxo-rapamycin was prepared as described by Luengo, J.I. et al. 1994 Tetrahedron Lett. 35, 6469-6472).

10 **Example 12. Rapalog-dependent transcriptional activation in transiently transfected cells:**

Rapalogs may be assayed for their ability to dimerize FKBP and FRB fusion proteins using a transcription read out as follows. Constructs encoding rapalog-dependent transcription factor fusion proteins are introduced into cells which  
15 contain, or are engineered to contain, a reporter gene linked to transcriptional regulatory DNA permitting reporter gene expression following rapalog-dependent dimerization of the transcription factor fusion proteins. The transcription factor fusion proteins include (a) an FKBP fusion protein containing, as a heterologous effector domain, a DNA binding domain (DBD) and (b) an FRB fusion protein  
20 containing, as a heterologous effector domain, a transcription activator domain. The FKBP and/or FRB domains may contain naturally occurring or non-naturally occurring peptide sequence. The presence of a rapalog which is capable of mediating dimerization of the two fusion proteins leads to expression of the reporter gene. The level of reporter observed is indicative of the activity of the rapalog as a dimerizer.  
25 Use of a target gene of interest in place of a reporter gene renders this a regulated gene expression system for use in cells grown in culture or in whole organisms.

We have made use of such a system as follows: HT1080 cells (ATCC CCL-121), derived from a human fibrosarcoma, were grown in MEM supplemented with non-essential amino acids and 10% Fetal Bovine Serum. Cells plated in 24-well  
30 dishes (Falcon,  $6 \times 10^4$  cells/well) were transfected using Lipofectamine under conditions recommended by the manufacturer (GIBCO/BRL). A total of 300 ng of the following DNA was transfected into each well:

- (a) 100 ng ZFHDx12-CMV-SEAP reporter gene (reporter gene linked to 12 recognition sites for the ZFHD1 DNA-binding domain),  
35 (b) 2.5ng pCGNN-ZFHD1-3FKBP or other DNA binding domain fusion (fusion protein comprising 3 FKBP domains and one ZFHD1 domain),

- (c) 5 ng pCGNN-1FRB-p65(361-550) (fusion protein comprising an FRB domain and a p65 transcription activation domain) and
- (d) 192.5 ng pUC118.

In some experiments, pCGNN-1FRB(T2098L)-p65(361-550) was used in place of pCGNN-1FRB-p65(361-550) to generate an FRB fusion protein containing an engineered FRB domain. In control experiments where the DNA binding domain or activation domain were omitted, an equivalent amount of empty pCGNN expression vector was substituted. For detailed information on the assembly and use of constructs including those mentioned herein, see WO 96/41865 (Clackson et al), especially the Examples therein (which are specifically incorporated by reference herein). Following lipofection (for 5 hours) 500  $\mu$ l medium containing the indicated amounts of rapalog was added to each well. After 24 hours, medium was removed and assayed for SEAP activity as described (Spencer et al, Science 262:1019-24, 1993). Human fibrosarcoma cells transiently transfected with a SEAP target gene and plasmids encoding representative ZFHD-FKBP- and FRB-p65-containing fusion proteins exhibited rapalog-dependent and dose-responsive secretion of SEAP into the cell culture medium. SEAP production was not detected in cells in which one or both of the transcription factor fusion plasmids was omitted, nor was it detected in the absence of added rapalog. As shown in Figure 1, cells transfected with wild-type FKBP and FRB constructs exhibited SEAP production at dimerizer concentrations as low as 1 nM. Figure 2 illustrates preferential stimulation of SEAP production in cells expressing a mutant FRB (T2098L, Figures 2B and 2D; T2098F, Figure 2E) as compared to wild-type (Figures 2A and 2C). Similar results have been obtained when the same transcription factors were used to drive rapalog-dependent activation of an hGH target gene or a stably integrated version of the SEAP reporter gene made by infection with a retroviral vector.

#### Example 13: Mutagenesis and phage display to generate modified Ligand-Binding Domains complementary to various rapalogs

##### A. Engineered FKBP and FRB domains

We have designed and prepared recombinant DNA constructs encoding the fusion proteins tabulated below which bear illustrative modified ligand-binding domains. Except a otherwise stated, mutants were generated using oligonucleotide-mediated site-directed mutagenesis according to standard methods (Kunkel, T.A., Bebenek, K. and McClary, J. 1991. Meth Enzymol 204, 235-139), and confirmed by dideoxy sequencing.

**Fusion Proteins containing modified FKBP domains**

(F36V hFKBP12)—p65

5 (F36V hFKBP12)—(F36V hFKBP12)—p65

(F36V hFKBP12)—(F36V hFKBP12)—(F36V hFKBP12)—p65

(F36M hFKBP12)—p65

(F36M hFKBP12)—(F36M hFKBP12)—p65

(F36M hFKBP12)—(F36M hFKBP12)—(F36M hFKBP12)—p65

10 (F36V hFKBP12)—ZFHD1

(F36V hFKBP12)—(F36V hFKBP12)—ZFHD1

(F36V hFKBP12)—(F36V hFKBP12)—(F36V hFKBP12)—ZFHD1

(F36M hFKBP12)—ZFHD1

(F36M hFKBP12)—(F36M hFKBP12)—ZFHD1

15 (F36M hFKBP12)—(F36M hFKBP12)—(F36M hFKBP12)—ZFHD1

myr-(F36V hFKBP12)—(F36V hFKBP12)—Fas

myr-(F36M hFKBP12)—(F36M hFKBP12)—Fas

myr-(F36A hFKBP12)—(F36A hFKBP12)—Fas

myr-(F36S/F99A hFKBP12)—(F36S/F99A hFKBP12)—Fas

20

-

**notes:**

1. "hFKBP12" indicates amino acids 1-107 of human FKBP12 referred to previously

2. "p65" indicates residues 361-550 of p65

25

3. "Fas" indicates residues 175-304 of human Fas

4. "ZFHD1" is as described elsewhere

5. "myr" indicates the src myristoylation sequence

6. mutations are indicated using the previously described convention

30

We have also prepared constructs encoding the following FRB fusion proteins:

Fusion Proteins containing modified (hFRAP) FRB domains

(T2098A FRB)---p65  
 (T2098N FRB)---p65  
 (D2102A FRB)---p65  
 (Y2038H FRB)---p65  
 (Y2038L FRB)---p65  
 (Y2038A FRB)---p65  
  
 (F2039H FRB)---p65  
 (F2039L FRB)---p65  
 (F2039A FRB)---p65  
 (K2095S/D2096N/T2098N FRB)---p65  
 (TOR2 FRB)---p65

notes:

1. "p65" indicates p65 residues 361-550, as above
2. "FRB" indicates the 89 amino acid FRB of human FRAP
3. "TOR2 FRB" indicates amino acids 1961-2052 of *S. cerevisiae* TOR2

Yeast and *Candida* FRBs, modified by analogy to the modified hFRAP FRB domains discussed herein, may also be prepared by substitution of a codon for a different amino acid in place of one or more of the two conserved Phe residues and the conserved Asp and Asn residues within each of their FRB domains. Illustrative modified FRB domains derived from TOR 1 and TOR2, include the following:

| Modified TOR1 and TOR2 FRB Domains  |        |
|---|--------|
| TOR1  | TOR2   |
| F1975H  | F1978H |
| F1975L  | F1978L |
| F1975A  | F1978A |
| F1975S  | F1978S |
| F1975V  | F1978V |
| F1976H  | F1979H |
| F1976L  | F1979L |
| F1976A  | F1979A |
| F1976S  | F1979S |
| F1976V  | F1979V |
| D2039A  | D2042A |
| N2035A  | N2038A |
| N2035S  | N2038S |
| These modified TOR1 and TOR2 FRBs are designed for use with rapalog<br>containing C7 substituents |        |

#### B. Testing rationally designed FKBP mutants for binding to rapalogs

An expression vector based on pET20b (Novagen) was constructed using standard procedures that expresses FKBP preceded by a hexahistidine tag and a portion of the H. influenza hemagglutinin protein that is an epitope for the monoclonal antibody 12CA5. The sequence of the protein encoded by this vector is as follows:

```

His6  HA tag____  FKBP-->
MHHHHHHYPYDVPDYAAMAHMGVQVETISPGDGRTPPKRGQTCVVHYTGMLEDGKKFDSSR
DRNKPFFKFMGLGQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDV
ELLKLE

```

To generate expression vectors for FKBP mutants at rapamycin contact residues, oligonucleotide-mediated site-directed mutagenesis was performed on the single-stranded form of the vector prepared from E.coli CJ236, as described (Kunkel, T.A., Bebenek, K. and

McClary, J. 1991. *Meth Enzymol.* 204, 235-139). Mutants were confirmed by dideoxy sequencing. Mutant proteins were expressed in *E.coli* BL21(DE3) (Novagen) as described (Wiederrecht, G. et al. 1992. *J. Biol. Chem.* 267, 21753-21760), and purified to homogeneity as described (Cardenas, M.E. et al. 1994. *EMBO J.* 13, 5944-5957).

- 5 Using this protocol the following mutant human FKBP12 proteins were generated, using the indicated oligonucleotide primers (mutated bases in upper case; 5'→3'):

Mutants designed for binding to C24 rapalogs:

|    |          |                             |     |
|----|----------|-----------------------------|-----|
| 10 | Phe46His | agcataaacttaTGgggcttgtttctg | (1) |
|    | Phe46Leu | agcataaacttTaagggcttgtttctg | (2) |
|    | Phe46Ala | agcataaacttaGCgggcttgtttctg | (3) |
|    | Phe48His | ttgcctagcataTGcttaaagggcttg | (4) |
|    | Phe48Leu | ttgcctagcatTaacttaaagggcttg | (5) |
| 15 | Phe48Ala | ttgcctagcataGCcttaaagggcttg | (6) |
|    | Glu54Ala | cctcggatcaccGCctgttgcttag   | (7) |
|    | Val55Ala | cagcctcggatcGCctcctgttgcc   | (8) |

Mutants designed for binding to C13/C14 rapalogs:

|    |                     |                               |                  |
|----|---------------------|-------------------------------|------------------|
| 20 | Phe36Ala            | ccgggaggaatcGGCttttttccatcttc | (9)              |
|    | Phe36Val            | ccgggaggaatcGACttttttccatcttc | (10)             |
|    | Phe36Ser            | ccgggaggaatcAGAttttttccatcttc | (11)             |
|    | Phe36Met            | ccgggaggaatcCATttttttccatcttc | (12)             |
| 25 | (Phe36Met+Phe99Ala) | aagctccacatcGCCgacgagagtggc   | (13) + primer 12 |
|    | (Phe36Met+Phe99Gly) | aagctccacatcGCCgacgagagtggc   | (14) + primer 12 |
|    | (Phe36Ala+Phe99Ala) | primer 9 + primer 13          |                  |
|    | (Phe36Ala+Phe99Gly) | primer 9 + primer 14          |                  |
|    | Tyr26Ala            | caagcatcccgtgGCgtgcaccacgtag  | (15)             |
| 30 | Asp37Ala            | tcccgaggagaaGCaaattttttccatc  | (16)             |

Mutant designed for binding to C28/C30 rapalogs:

|    |          |                           |      |
|----|----------|---------------------------|------|
| 35 | Glu54Ala | cctcggatcaccGCctgttgcttag | (17) |
|----|----------|---------------------------|------|



To assay the relative binding affinity of rapamycin and rapalogs to FKBP mutants, a competitive fluorescence polarization (FP) assay is used that relies on the retention of FK506 (and hence probe) binding affinity by the mutants. The procedure is identical to that described in Example 12 except that a direct binding assay is first performed to determine the dilution (concentration) of mutant FKBP to use in the competition reactions in order to obtain sub-saturation. Serial dilutions of mutant FKBP are made in FP buffer (Example 12) in 100µl volumes in Dynatech micro-fluor plates, and then 100µl of 10 nM AP149. (probe) in [FP buffer + 2% ethanol] added to each well. Equilibration and plate reading are as in Example 12. A plot of mP units vs concentration of FKBP mutant is fit to following equation:

$$y = M3 + (((x + M1 + M2) - \sqrt{((x + M1 + M2)^2 - (4 * x * M1))}) / (2 * (M1))) * (M4 - M3)$$

and the final mutant concentration/dilution at which 90% of probe is specifically bound is determined by interpolation. This final concentration is then used in a competition FP assay carried out as in Example 12, with 2 x the final concentration of mutant replacing 11.25 nM FKBP in the protocol. Instead of 90% saturation, 75% can be selected to impart greater sensitivity to the competition assay. Serial dilutions of rapamycin analogs are used as competitor and the results are expressed as IC50 for each rapalog binding to each mutant.

### C. Testing rationally designed FRB mutants for binding to FKBP-rapalog complexes

A NcoI-BamHI fragment encoding residues 2021-2113 (inclusive) of human FRAP was generated by PCR with primers 28 and 29 (below), and cloned into a derivative of pET2Cb(+) (Novagen) in which the NdeI site is mutated to NcoI, to create pET-FRAP(2021-2113). Single-stranded DNA of this vector was used as a template in site-directed mutagenesis procedures, as described above, to generate vectors encoding FRAPs mutated at rapamycin contact residues. Mutants were confirmed by dideoxy sequencing. Mutants were then amplified by PCR using primers (30 and 31) that append XbaI and SpeI sites, and cloned into XbaI-SpeI digested pCGNN-FRB-p65(361-550) (Example 7) to generate a series of constructs directing mammalian expression of chimeric proteins of the form E-N-mutant FRAP(2021-2113)-p65(361-550), where E indicates HA epitope tag and N indicates nuclear localization sequence. Constructs were verified by restriction digestion and dideoxy sequencing.

Using this procedure the following constructs encoding candidate mutant FRAPs for binding to C7 rapalogs, each fused to the p65(361-550) activation domain, were generated using the indicated oligonucleotide primers (mutated bases in upper case; 5'→3'):

|    |  |   |
|----|--|---|
| 5  | Tyr2038His                                 | cctttccccaagtGcaaacgagatgc (18)         |
|    | Tyr2038Leu                                 | cctttccccaagAGcaaacgagatgc (19)         |
|    | Tyr2038Ala                                 | cctttccccaagGCcaaacgagatgc (20)         |
|    | Phe2038His                                 | gttcctttccccAtGgtacaaacgagatg (21)      |
|    | Phe2038Leu                                 | gttcctttccccTaagtacaaacgagatg (22)      |
| 10 | Phe2038Ala                                 | gttcctttccccGCgtacaaacgagatg (23)       |
|    | Thr2098Ala                                 | gtcccaggcttggGCgaggtccttgac (24)        |
|    | (Lys2095Ser+Asp2096Asn+Thr2098Asn)<br>(25) | gtcccaggcttggTTgaggITcGAgacattccctgattc |
|    | Thr2098Asn                                 | gtcccaggcttggTTgaggtccttgac (26)        |
| 15 | Asp2102Ala                                 | catgataatagaggGCccaggcttgggtg (27)      |

To assay the relative binding affinity of these mutants for complexes of FKBP with rapamycin and various rapalogs, each construct is transiently co-transfected into human HT1080B14 cells, as described in Example 8. Following transfection, serial dilutions of rapamycin or rapalog are added to the culture medium. After 24 hours, SEAP activity is measured as described in Example 8; the potency of SEAP activation at various rapalog concentrations is proportional to the affinity of the FRAP mutant for the complex between FKBP and the rapalog.

25

PCR primers (restriction sites upper case; 5'→3'):

gcacCCATGGcaatccctctggcatgagatgtggcatgaaggcctggaag (28)

cgtgaGGATCCtactttgagattcgtcggaacac (29)

5 gcacTCTAGAatcctctggcatgagatgtggcatgaaggcctggaag (30)

ggctcGGATCCctaataACTAGTctttgagattcgtcggaacacatg (31)

D. Functional display of FKBP and FRB domains on filamentous bacteriophage: one approach to selection as an alternative to rational design of modified domains

10

A phage display system for the display and selection of mutant FKBP and FRB domains is disclosed in detail in WO 96/41865 (Clackson et al), including vector construction, preparation of His6-flag-FKBP, pCANTAB-AP-FKBP, Binding enrichments, Primers, the Sequence of pCANTAB-AP-FRAP(2015-2114) and pCANTAB-AP-FKBP, the synthesis of  
15 biotinylated FK506 for affinity enrichment studies, functional FKBP display by competitive ELISA using biotinylated FK506, generation of a library of mutant FKBP on phage targeted to the C13 and C14 positions of rapamycin and library sorting.

20

#### Example 14: Rapamycin-Dependent Activation of Signal Transduction

25

Many cellular receptors can be activated by aggregation, either by their physiological ligand or by anti-receptor antibodies. Additionally, the aggregation of two different proteins can often trigger an intracellular signal. Rapamycin and its analogs may be used to  
25 trigger activation of a receptor effector domain by oligomerizing chimeric proteins, one of which contains one or more FKBP and an effector domain and the other of which contains one or more FRAP domains and an effector domain. This scheme is illustrated in Figure T1(a). While both proteins are shown anchored to the membrane, a single one could be membrane anchored, and addition of rapamycin or analog would recruit the second protein to  
30 the membrane via dimerization. Membrane anchoring may be effected through a transmembrane protein anchor or through lipid modification of the protein(s), such as myristoylation. The same effector domain may be present on both proteins, or different protein domains that interact functionally may be used, such as a protein kinase and a protein kinase substrate. Alternatively, a second effector may serve to inhibit the activity  
35 of the first effector.

We note that in some embodiments, the chimeric proteins are mixed chimeras, discussed previously, and contain FKBP and FRAP domains together with the heterologous effector domain. Oligomerization of a single mixed chimera may also be used to activate signal transduction, as shown in Figure T1(b). Here rapamycin is shown to dimerize two identical copies of the protein. Reiteration of the FKBP and FRAP domains permits higher multiples to occur, subject to geometric constraints.

Two examples of the use of rapamycin in signal transduction are to trigger receptor tyrosine kinase activation and to trigger apoptosis via Fas activation, both of which are discussed below. Unless otherwise mentioned all DNA manipulations were performed following standard procedures (F.M. Ausubel et al., Eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1994) and all protein protocols were performed following standard procedures (Harlow, E. and Lane, D. 1988. Antibodies, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.). All PCR products used to make constructs were confirmed by sequencing.

#### A. Rapamycin-inducible receptor tyrosine kinase activation.

##### 1. Construction of pCM, an expression vector containing a myristylation signal.

A XbaI-Myr-BamHI cassette, obtained by annealing oligonucleotides 1 and 2, was digested with XbaI/BamHI and cloned into the XbaI/BamHI site of the pCG expression vector (Tanaka, M. and Herr, W. 1990. Cell 60: 375-386) to create pCGM. (For oligonucleotide sequences, see (7) below). This oligonucleotide cassette consists of an inframe XbaI site followed by sequence encoding for the first 15 amino acids residues of c-Src tyrosine kinase that has been shown to allow myristoylation and target protein to the plasma membrane (Cross et al., 1984. MCB. 4:1834-1842). The myristoylation domain is followed by an inframe SpeI site and stop codons. The XbaI site in the pCG vector is placed such that it adds two amino acids between the initiating Met and the sequence cloned. Since the spacing between the initiating Met and the myristylated Gly is crucial for membrane localization of c-Src (Pellman et al. 1985. PNAS. 82: 1623-1627) the XbaI site following the ATG in pCGM was deleted by site directed mutagenesis following manufacturers protocol (Muta-Gene, BioRad). To facilitate future cloning steps the SpeI site in the myristylation cassette was mutated to a XbaI site. Single stranded uracil-DNA of pCGM was prepared and the mutagenesis was carried out using both oligonucleotide 3 (to delete the XbaI site following ATG and add an EcoRI site 5' to ATG) and oligonucleotide 4 (to change the SpeI site following the myristylation domain to a XbaI site). The resulting sequence surrounding the ATG of the pCM vector was confirmed by sequencing using oligonucleotide 5 (see sequence 1, (8) below).

2. Addition of FKBP and an epitope tag to pCM generates pCMF1/2/3.HA.

A SpeI-HA-BamHI cassette was prepared by annealing complementary oligonucleotides (oligonucleotides 6 and 7). This cassette has an inframe SpeI site followed by nine amino acids of *H. influenzae* hemagglutinin gene that is recognized by the monoclonal antibody 12CA5, stop codons and a BamHI site. The SpeI-HA-BamHI cassette was sub cloned into the SpeI/BamHI site of pCGNNF1, pCGNNF2 and pCGNNF3. Subsequently, the 1/2/3 copies of FKBP fused with HA epitope was sub cloned as an XbaI/BamHI fragment into pCM. The resulting plasmid (pCMF1/2/3.HA) has the following features: myristylation domain; an inframe XbaI site; one/two/three copies of FKBP; an inframe SpeI site; a HA epitope tag; and stop codons.

3. Addition of FRBs and an epitope tag to pCM generates pCMFR1/2/3.Flag.

A SpeI-Flag-BamHI cassette can be prepared by annealing complementary oligonucleotides (oligonucleotides 8 and 9). This cassette has the same features as the SpeI-HA-BamHI cassette described above with the exception that the inframe SpeI site is followed by sequence that codes for eight amino acids (DYKDDDDY) (Hopp et al., 1988. Biotech. 6: 1205-1210) that is recognized by a monoclonal antibody anti-FLAG.M2 (Kodak Scientific Imaging Systems). The SpeI-Flag-BamHI cassette is sub cloned into the SpeI/BamHI site in pCGNN-1FRB, pCGNN-2FRB and, pCGNN-3FRB. Subsequently 1/2/3 copies of FRB domain-Flag epitope fusions are sub cloned as a XbaI/BamHI fragment into pCM. The resulting plasmid (pCMFR1/2/3.Flag) has the following features: myristylation domain; an inframe XbaI site; one/two/three copies of FRB; an inframe SpeI site; a Flag epitope tag; and stop codons.

4. Fusion of FKBP and FRB constructs to receptor tyrosine kinase cytoplasmic domain

The cytoplasmic domain of receptor tyrosine kinase of choice (e.g., EGFR, erbB-2, PDGFR, KDR/Flk-1, Flt-1) is PCR amplified with inframe 5'XbaI and 3' SpeI sites. The PCR product may be subcloned either into the inframe XbaI site such that the XbaI site is restored, or into the inframe SpeI site such that the SpeI site is restored in pCMFR series or pCMFseries vectors (see above). As a result, the FKBP/FRB domain(s) can be placed either C-terminal or NH2-terminal to the cytoplasmic domain of the receptor tyrosine kinase. The vectors are constructed such that (i) the cytoplasmic domain of a given receptor is fused to both FKBP and FRB (for e.g., EGFR cytoplasmic domain fused to either FKBP or FRB) or (ii) can be constructed such that cytoplasmic domains of two different receptors are fused to FKBP and FRB (for e.g., EGFR cytoplasmic domain fused to FKBP and erbB-2 cytoplasmic

domain fused to FRB). In the former case (i) addition of the drug, rapamycin, will induce the formation of homodimers (e.g., EGFR/EGFR) while, in the latter (ii) addition of the drug will induce heterodimer (e.g., EGFR/erbB-2) and result in activation of the signal transduction cascade.

5

#### 5. Testing the constructs

To test the ability of rapamycin or analog to induce dimerization of FKBP- and FRB-receptor cytoplasmic domain fusions, the constructs of choice (e.g., pCMVEGFR-FR1 and pCMVEGFR-F1) are cotransfected into Cos-1 cells by lipofection (Gibco BRL). Three days after transfection the cells are induced with rapamycin and lysed in lysis buffer (1% Triton X-100; 50mM Tris,cl pH8.0; 150mM NaCl; 5mM NaF; 1mM sodium ortho vanadate; 10ug/ml aprotinin; 10ug/ml leupeptin). The fusion proteins from rapamycin-treated and untreated cell lysates are immunoprecipitated with anti-Flag and 12CA5 antibodies and immunoblotted with anti-phosphotyrosine antibody. The choice of cell type; the amount of DNA transfected; the concentration of rapamycin used and the duration of drug treatment are varied to achieve optimal results.

15

#### 6. Rapamycin-inducible cell growth

A selected mammalian cell line (e.g., NIH3T3) is cotransfected with constructs encoding for FRB and FKBP fusion proteins (e.g., pCMVEGFR-FR1 and pCMVEGFR-F1) and stable cell lines expressing the fusion proteins are established. To determine whether rapamycin-inducible activation of receptor cytoplasmic domain will induce cell proliferation, stable cell lines expressing the fusion proteins are grown either in the presence or absence of rapamycin and the changes in cell growth rate are determined by routine procedures (e.g., by monitoring cell number; by determining the <sup>3</sup>H thymidine incorporation rate, etc.). The choice of receptor tyrosine kinase; the type of receptor activation (homodimer vs. heterodimer) may be chosen to obtain optimal results.

25

#### 7. Oligonucleotide sequences

30

- 1: CATGTCTAGAGGGAGTAGCAAGAGCAAGCCTAAG GACCCCAGCCAG  
CGCACTAGTTAAGAATTCTGATGAT CAGCGGATCCTAGC
- 2: GCTAGGATCCGCTGATCATCAGAATTCTTAAGTAGTG  
CGCTGGCTGGGGTCCTTAGGCTTGCTTTGCTACTCC CTCTAGACATG
- 35 3: CGCCTTGTAAGAATTCGCGGTATGGGGAGTAGCAAGA
- 4: CCCAGCCAGCGCTCTAGATAAGAATTCTGA

5: AAGGGTCCCCAAACTCAC  
 6: GCATGACTAGTTATCCGTACGACGTACCAGACT  
     ACGCATAAGAAAAGTGAGGATCCTACGG  
 7: CCGTAGGATCCTCACTTTTCTTATGCGTAGTCTGGT  
 5     ACGTCGTACGGATAACTAGTCATGC  
 8: CCGTAGGATCCTCACTTTTCTTAATAATCGTCATCG  
     TCTTTGTAGTCACTAGTCATGC  
 9: GCA<sup>T</sup>GACTAGTGACTACAAAGACGATGACGATTA  
     TTAAGAAAAGTGAGGATCCTACGG

10

8. Sequence 1:

                          M   G   S   S   K   S   K   P   K  
 CGC CTT GTA GAA ttc GCG CGT ATG ggg agt agc aag agc aag cct aag

15

          D   P   S   Q   R   S   R   stop                   stop  
 gac ccc agc cag cgc tct aga taa gaa ttc tga tga tca gcG GAT CCT

20 GAG AAC T

The modified sequences are in lowercase bold and the initiating ATG is underlined.  
 Sequences in uppercase are from the parental pCG backbone.

## 25 B. Rapamycin-inducible apoptosis

The ability to control Fas activation and trigger apoptosis via a small molecule has applications both in gene therapy, where it may be used to selectively eliminate engineered cells, and in experimental systems. The proteins described here are anchored to the membrane via the low affinity NCF receptor, also called p75. It should be appreciated,  
 30 however, that another protein anchor could be readily substituted. p75 is useful experimentally because of the availability of antibodies to its extracellular domain, and its lack of high affinity interaction with any identified ligand (Bothwell, M. 1995. Annu. Rev. Neurosci. 18:223-253).

## 35 1. 2-Protein Rapamycin-Regulated Fas Activation

## (a) Construction of the p75 vector

Vectors to direct the expression of FRAP-Fas fusion proteins containing the extracellular and transmembrane domain of the low affinity NGF receptor (also known as p75) were

derived from the mammalian expression vector pJ7W (Morgenstern, J.P. and Land, H. 1990. Nucleic Acids Res. 18:1068), modified by substitution of a pUC backbone for the original pBR backbone using standard methods. We call this vector pA7W. Inserts cloned into the polylinker sites of this plasmid are transcribed under the control of the simian CMV promoter and enhancer sequences. The polylinker follows the CMV sequence with HindIII-SalI-XbaI-BamHI-SmaI-SstI-EcoRI-ClaI-KpnI-BglII. Any mammalian expression vector with suitable cloning sites and promoter could be substituted.

A restriction fragment encoding a fragment of p75 flanked by HindIII and XbaI sites was generated by PCR using primers J1 (5') and J2 (3'), based on the sequence of p75 (Johnson, D., Lanahan, A., Buck, C.R., Shegal, A., Morgan, C., Mercer, E., Bothwell, M., Chao, M. 1986. Cell 47:545-554). The original source of the PCR template was a clone derived from a human brain library, using primers similar to J1 and J2 but with different restriction sites. The 5' end of the resulting fragment contains a HindIII site followed by an EcoRI site, a Kozak sequence and the initiation of p75 coding sequence (amino acid 1). The 3' end generated encodes the receptor sequence up to and including amino acid 274, 2 amino acids past the predicted membrane spanning sequence, followed by an XbaI site. Analogous portions of other transmembrane receptors can be substituted for this fragment. The PCR product was subcloned as a HindIII-XbaI fragment into HindIII-XbaI cut pA7W, generating pA7Wp75. The construct was verified by restriction analysis and DNA sequencing.

(b) Addition of Fas to pA7Wp75

XbaI-SpeI fragments encoding Fas amino acids 206-304 (FasS) and Fas amino acids 206-319 (FasL) were made by PCR and subcloned into pA7Wp75 cut with the same enzymes. The primers used were J3 (5') and J4 or J5 (3'). J5 generates a fragment of Fas that ends beyond its termination codon; when cut with SpeI, the nucleotides encoding the terminal 15 aa of Fas are removed to give a truncated form of intracellular Fas we call FasS. Removal of these 15 aa increases the activity of Fas in some cell types (Itoh, N., and Nagata, S. 1993. J. Biol. Chem 268:10932). Primer J4 replaces the natural termination codon of Fas with a SpeI site, and also mutates the original SpeI site contained in Fas, generating FasL. The plasmids generated from subcloning these fragments are pA7Wp75-FasS and pA7Wp75-FasL, respectively. These construct were verified by restriction analysis and DNA sequencing. To attach an epitope tag to these inserts, the XbaI-SpeI Fas fragments were isolated and ligated into the XbaI-SpeI cut backbone of pCMF1/2/3.HA, plasmids described above which encode an epitope tag of 9 amino acids from the H. influenza haemagglutinin protein (E) 3' to the SpeI site, followed by a BamHI site. Cutting the resultant plasmid with XbaI and



BamHI generated fragments encoding Fas followed by the epitope tag (designated E for these constructs).

(c). p75-FRAP-Fas-epitope fusion proteins: addition of FRAP-containing fragments to pA7Wp75-FasSE and pA7Wp75-FasLE to generate p75-FRAPx-FasSorLE and p75-FasSorL-FRAPxE

The XbaI-SpeI fragments containing a portion of FRAP are described previously in this document. These XbaI-SpeI fragments were inserted into either the XbaI site directly after the p75 coding sequence to generate p75-FRAPx-FasSorLE or into the SpeI site directly after the Fas fragment to generate p75-FasSorL-FRAPxE. Alternatively, more than one FRAP fragment is subcloned in, either as a FRAPn fragment, or by sequential subcloning of XbaI-SpeI fragments into the Spe I site available after subcloning the first FRAP into either XbaI or SpeI. Thus the final series of vectors encodes (from the N to the C terminus) p75 extracellular and transmembrane sequence, one or more FRAP-derived domains fused N- or C-terminally to one or more Fas intracellular domains, and an epitope tag.

(d) p75-FKBP-Fas fusion proteins: addition of FKBP-containing fragments to pA7Wp75-FasSE and pA7Wp75-FasLE to generate p75-FKBPn-FasSorL or p75-FasSorL-FKBPn

The XbaI-SpeI fragments containing one or more FKBP's have been described elsewhere in this document. These fragments were inserted into either the XbaI site directly after the p75 coding sequence to generate p75-FKBPn-FasSorL or into the SpeI site directly after the Fas fragment to generate p75-FasSorL-FKBPn. Thus the final series of vectors encodes (from the N- to the C-terminus) p75 extracellular and transmembrane sequence, one or more FKBP's fused N- or C-terminally to one or more Fas intracellular domains, and an epitope tag.

#### (e) Assay of Rapamycin-Mediated Fas Activation

The ability of expression of a protein containing Fas and FRAP domains and a protein containing Fas and FKBP domains to activate Fas and trigger cell death upon addition of rapamycin can be tested in either transiently or stably transfected cells.

For transient transfections, the two plasmids to be tested are cotransfected into a cell line such as HT1080 by a standard method such as lipofection, calcium phosphate precipitation or electroporation. One or more days after transfection, cells are treated with no addition or one or more concentrations of rapamycin or one or more concentrations of a dimerizing agent such as FK1012. The FK1012 serves as a positive control that the FKBP-Fas construct is functional. Several hours to 1 day later, the cells are monitored for response by one of several methods. Cell lysates were prepared by conventional means and used to

generate Western blots that are probed with antibody directed against HA or against the extracellular domain of p75. Alternatively, cells can be assayed by collection in isotonic solution plus 10 mM EDTA, stained with anti-p75 monoclonal antibody and labeled secondary antibody, and the positive cells measured by FACS. A decrease in either Western  
5 blot signal or FACS signal upon treatment indicates successful induction of cell death (or decrease in protein expression). In addition, commercially available kits can be used to monitor apoptosis.

To stably transfect cells, a vector encoding a selectable marker such as neomycin resistance is cotransfected along with the plasmids described. Two to three days after  
10 transfection, cells are plated into G418 and the resistant population or clones are isolated by standard means. These populations can then be monitored directly for induction of apoptosis by treatment with dimerizer followed by cell counting or other measure of cell viability.

An alternative means of generating stable cell lines expressing the constructs of interest is to subclone the inserts into a retroviral vector. The inserts are excisable with Eco RI to  
15 facilitate this subcloning. The vector is then used to make transducing supernatants by a packaging cell using conventional methods.

## 2. Single Protein Rapamycin-Regulated Fas Activation

### (a). Construction of FKBP-FRAP chimeric fragments FKBP-FRAP fusion constructs for rapamycin-dependent homodimerization of Fas intracellular domain

5

#### i. Structure-assisted design

In order to design molecules containing both FRAP and FKBP domains that are capable of rapamycin-dependent homodimerization, the three dimensional structure of the ternary complex between human FKBP12, rapamycin, and a portion of human FRAP encompassing the minimal FRB domain may be considered. Requirements for homodimerization of two molecules of fusion proteins containing FRAP, FKBP and Fas moieties include (i) sufficient length and flexibility of the polypeptide to accommodate the distortions necessary for the FRAP-FKBP interaction to occur between molecules tethered at the membrane, while preserving the ability of aggregated Fas to transduce a signal; and (ii) prevention or minimization of intramolecular dimerization by rapamycin, an event expected to be highly entropically favored due to the chelate effect, and therefore to prevent the desired intermolecular molecular dimerization.

15

Structural considerations led us to the following design preferences for the fusion constructs:

20

(i) FRB and FKBP should be joined with a polypeptide linker sufficiently short that intramolecular dimerization is sterically prevented. The currently preferred configuration is FRAP-FKBP as the C-terminus of FRAP and the N-terminus of FKBP are distant, allowing a long linker (>ten amino acids) that should still prevent intramolecular dimerization yet afford flexibility.

25

(ii) This FRAP-FKBP 'cassette' can be present membrane-proximally (i.e. with Fas domain(s) added to the C-terminus), or membrane distal (with the Fas domain membrane-proximal and the FRAP-FKBP cassette appended C-terminally).

30

(iii) A long linker should be present N-terminal to the FRAP-FKBP domains, to allow for the structural distortions implied by dimerization at the membrane or if the domains are added C-terminally. Again a N-terminal location of FRAP is preferred as this long linker can then comprise natural FRAP sequence from the region N-terminal to the FRB domain, minimizing the immunogenicity of the chimeric protein.

35

(iv) Optimal linker lengths and fusion positions for a given protein should be confirmed empirically.

5 A series of 12 fusions of FKBP and FRAP, designated T1-T12, was designed. Nine were N-FRAP-FKBP-C fusions including between 13, 23 or 33 amino acids N-terminal to Arg2018 (the N-terminal linker), and 4, 7 or 10 residues separating the two proteins. The remaining three were N-FKBP-FRAP-C fusions interposing 3, 0 or -4 residues of FRAP sequence between FKBP Glu107 and FRAP Arg2018.

10 (ii) Construction

The twelve fusions were made as XbaI-BamHI cassettes that could be cloned directly as a single fragment, using the three-primer PCR splicing method (Yon, J. and Fried, M. 1989. Nucleic Acids Res. 17, 4895). Cloning in this way avoided the introduction of restriction sites between the genes that would encode foreign sequence and alter the length of the linker. A  
15 mixture of 1 ng each of pCGNN-1FRAPi and pCANTAB-AP-FKBP was amplified using Pfu polymerase with 1  $\mu$ M each of two outer primers (A and C), in the presence of 0.01  $\mu$ M of a single 'splice' oligo (B) complementary to both genes that directs the desired fusion. The primers used are tabulated below:

20

| #  | construct                | oligos |     | N-term* |      | linkert |
|----|--------------------------|--------|-----|---------|------|---------|
|    |                          | A      | B   | C       | (aa) | (aa)    |
|    | T1 FRAP(1985-2116)-FKBP  | 100    | 102 | 105     | 33   | 4       |
|    | T2 FRAP(1995-2116)-FKBP  | 93     | 102 | 105     | 23   | 4       |
| 5  | T3 FRAP(2005-2116)-FKBP  | 101    | 102 | 105     | 13   | 4       |
|    | T4 FRAP(1985-2119)-FKBP  | 100    | 103 | 105     | 33   | 7       |
|    | T5 FRAP(1995-2119)-FKBP  | 93     | 103 | 105     | 23   | 7       |
|    | T6 FRAP(2005-2119)-FKBP  | 101    | 103 | 105     | 13   | 7       |
|    | T7 FRAP(1985-2122)-FKBP  | 100    | 104 | 105     | 33   | 10      |
| 10 | T8 FRAP(1995-2122)-FKBP  | 93     | 104 | 105     | 23   | 10      |
|    | T9 FRAP(2005-2122)-FKBP  | 101    | 104 | 105     | 13   | 10      |
|    | T10 FKBP-FRAP(2014-2114) | 106    | 107 | 110     | -    | 3       |
|    | T11 FKBP-FRAP(2018-2114) | 106    | 108 | 110     | -    | 0       |
|    | T12 FKBP-FRAP(2021-2114) | 106    | 109 | 110     | -    | -4      |

\* Number of amino acids between the Arg encoded by the 5' XbaI site and FRAP Arg2018 (for fusions T1-T9)

+ Number of amino acids between FRAP Ser2112 and FKBP Gly1 (for fusions T1-T9); or between FKBP Glu107 and FRAP Arg 2018 (for fusions T10-T12)

PCR products were purified, digested with XbaI and BamHI, and ligated into XbaI-BamHI digested pCM. The constructs were verified by restriction analysis and DNA sequencing.

Primer sequences and the sequence of a representative FRB-FKBP construct: fusion T6 of FRAP (2005-→)-FKBP are disclosed in WO 96/41865 (p. 109).

(b) Addition of FRAP-FKBP chimeric inserts to pA7Wp75-FasSE and pA7Wp75-FasLE

Subcloning of T1 through T12 as XbaI-SpeI fragments into pA7Wp75-FasSE and pA7Wp75-FasLE linearized with XbaI generates p75TFasSorLE. Subcloning into pA7Wp75-FasLE linearized with SpeI generated p75TFasSorLT-E. These constructs are listed in Table 1 ((d) below).

(c) Alternative FRAP-Fas-FKBP constructs

Instead of the format of the chimeric fragments T1-T12, the single chain strategy could require a different orientation of domains for optimal activity. To this end, another series of

constructs was made in which FKBP and FRB are separated by a Fas fragment. The starting points for these constructs are pCMF1HA, pCMF2HA, and pCMF3HA. Similar to the strategy described above for the construction of chimeric transcription factors, FKBP and FRB fragments (described elsewhere in this document) were cloned into the pCM backbones as XbaI-BamHI fragments that included a SpeI site just upstream of the BamHI site. As XbaI and SpeI produce compatible ends, this allowed further XbaI-BamHI fragments to be inserted downstream of the initial insert. Additionally, cloning of an XbaI-SpeI fragment results in the addition of the fragment at the 5' end of the construct. The final p75-anchored construct was made by subcloning the XbaI-SpeI fragments shown in Table 1 ((d) below) into pA7Wp75-FasSE. A similar series is made by subcloning into pA7Wp75-FasLE. Insertion into vector cut with XbaI resulted in addition of the insert 3' to the p75 fragment. Insertion into this vector cut with SpeI resulted in addition of the insert 3' to the Fas fragment. Insertion into this vector cut with XbaI and SpeI resulted in addition 3' to the p75 fragment, and elimination of the Fas fragment originally in the vector. By using these three subcloning strategies, the following series of constructs was generated. Numerical subscripts define the number of times the domain is reiterated.

## (d) Table 1.

Code: N= p75 NGF receptor aa 1-274

FasS= Fas aa 206-304

FasL= Fas aa 206-319

5 K=FKBP aa 2-108

R=FRAP 2012-2113, but other boundaries can be substituted

E= HA epitope followed by termination codons as described in pCMF1/2/3.HA

| NAME | Xba I-Spe I<br>FRAGMENT<br>SUBCLONED | VECTOR SITE(S)<br>USED TO<br>SUBCLONE INSERT<br>INTO pA7Wp75-<br>FasSE | CONSTRUCT      |
|------|--------------------------------------|--|----------------|
| A1   | K2FasL                               | Spe I + Xba I  | NK2FasLE       |
| A2   | R                                    | Spe I  | NFasSRE        |
| A3   | R                                    | Xba I  | NRFasSE        |
| A4   | R2                                   | Spe I  | NFasSR2E       |
| A5   | R2                                   | Xba I  | NR2FasSE       |
| A6   | K2FasSR                              | Spe I  | NFasSK2FasSRE  |
| A7   | KFasSR                               | Spe I  | NFasSKFasSRE   |
| A8   | K2FasSR2                             | Spe I  | NFasSK2FasSR2E |
| A9   | KFasSR2                              | Spe I  | NFasSKFasSR2E  |
| A10  | T1                                   | Spe I  | NFasST1E       |
| A11  | T2                                   | Spe I  | NFasST2E       |
| A12  | T3                                   | Spe I  | NFasST3E       |
| A13  | T4                                   | Spe I  | NFasST4E       |
| A14  | T5                                   | Spe I  | NFasST5E       |
| A15  | T6                                   | Spe I  | NFasST6E       |
| A16  | T7                                   | Spe I  | NFasST7E       |
| A17  | T8                                   | Spe I  | NFasST8E       |
| A18  | T9                                   | Spe I  | NFasST9E       |
| A19  | T10                                  | Spe I  | NFasST10E      |
| A20  | T11                                  | Spe I  | NFasST11E      |
| A21  | T12                                  | Spe I  | NFasST12E      |
| A22  | K2FasSR                              | Xba I  | NK2FasSRFasSE  |
| A23  | KFasSR                               | Xba I  | NKFasSRFasSE   |

|     |          |               |                |
|-----|----------|---------------|----------------|
| A24 | K2FasSR2 | Xba I         | NK2FasSR2FasSE |
| A25 | KFasSR2  | Xba I         | NKFasSR2FasSE  |
| A26 | T1       | Xba I         | NT1FasSE       |
| A27 | T2       | Xba I         | NT2FasSE       |
| A28 | T3       | Xba I         | NT3FasSE       |
| A29 | T4       | Xba I         | NT4FasSE       |
| A30 | T5       | Xba I         | NT5FasSE       |
| A31 | T6       | Xba I         | NT6FasSE       |
| A32 | T7       | Xba I         | NT7FasSE       |
| A33 | T8       | Xba I         | NT8FasSE       |
| A34 | T9       | Xba I         | NT9FasSE       |
| A35 | T10      | Xba I         | NT10FasSE      |
| A36 | T11      | Xba I         | NT11FasSE      |
| A37 | T12      | Xba I         | NT12FasSE      |
| A38 | K2FasSR  | Spe I + Xba I | NK2FasSRE      |
| A39 | KFasSR   | Spe I + Xba I | NKFasSRE       |
| A40 | K2FasSR2 | Spe I + Xba I | NK2FasSR2E     |
| A41 | KFasSR2  | Spe I + Xba I | NKFasSR2E      |

(e) Termini/junction sequences of fragments, oligos and other details for construction of the inserts which were cloned in 3' to the myristoylation signal sequence as XbaI-BamHI or XbaI-SpeI fragments are disclosed in detail in WO 96/41865.

(f) Rapamycin-regulated apoptosis of stable transsfected human HT1080 cells in culture

XbaI-BamHI fragments from constructs A30 and A31 (d, table 1) were cloned into pCM to generate M30 and M31, constructs that direct the expression of MT5FasSE and MT6FasSE, where M denotes a myristoylation domain (see this example sections A.1. and A.8.) and other abbreviations are as described in d, table 1. EcoRI-BamHI fragments containing these expression cassettes were then cloned into the retroviral vector pSMTN3 (Example 7). Helper-free retroviruses containing this DNA were generated by transient co-transfection of 293T cells (Pear, W.S. et al. 1993. Proc. Natl. Acad. Sci. USA, 90, 8392-8396) with the constructs and a Psi(-) amphotropic packaging vector. HT1080 cells were infected with viral stock and selected with G418.



To assay apoptosis of the stably transfected pools of cells in response to rapamycin, cells were plated in a 96-well culture plates at 10000 cells/well. After an overnight incubation, serial dilutions of rapamycin were added, together with 50 ng/ml (final) actinomycin D, and incubation continued at 37°C and 5% CO<sub>2</sub> for approximately 20 hours. The media was removed and replaced with 100µl of media containing 10% alamar blue dye. Plates were incubated as before, and the extent of cell viability assessed periodically by spectrophotometric determination of OD at 570nm and 600nm on a microtiter plate reader. Typically reading was continued until control (untreated) wells are at OD 0.2-0.4 after subtraction of blank.

Survival of cells stably transfected with (a) M30 and (b) M31-expressing constructs is potentially reduced in the presence of rapamycin, in a dose-dependent manner. The extent of cell death is comparable to that of cells expressing a myristoylated (FKBP x 2)-Fas construct (as disclosed in PCT/US94/08008) treated with a synthetic FKBP homodimerizer AP1428. This system may be adapted for use with improved rapalogs of this invention, preferably with one or more mutations in the FKBP and/or FRB domains used.

• • • •

The full disclosure of each of the patent documents and scientific papers cited herein is hereby incorporated by reference. Those documents serve to illustrate the state of the art in various aspects of this invention. Numerous modifications and variations of the present invention should be apparent to one of skill in the art. Such modifications and variations, including design choices in selecting a heterologous action domain, improved rapalog, fusion protein design, DNA formulation, viral vector or other DNA delivery means, manner and route of transgene administration, etc. are intended to be encompassed by the scope of the invention and of the appended claims.

## Claims:

1. A method for multimerizing chimeric proteins in cells which comprises:

5

(a) providing cells which contain:

10

(i) a first recombinant nucleic acid encoding a first chimeric protein which binds to rapamycin or an analog thereof and which comprises at least one FKBP domain and at least one protein domain heterologous thereto, wherein the FKBP domain comprises a peptide sequence selected from:

15

- (1) a naturally occurring FKBP
- (2) a variant of a naturally occurring FKBP in which up to 10 amino acid residues have been deleted, inserted, or replaced with substitute amino acids,
- (3) an FKBP encoded by a DNA sequence capable of selectively hybridizing to a DNA sequence encoding an FKBP of (i) or (ii);

20

(ii) a second recombinant nucleic acid encoding a second chimeric protein which forms a complex with both (a) rapamycin or a rapamycin analog and (b) the first chimeric protein, and which comprises at least one FRB domain and at least one domain heterologous thereto, wherein the FRB domain comprises a peptide sequence selected from:

25

- (1) a naturally occurring FRB domain,
- (2) a variant of a naturally occurring FRB domain in which up to 10 amino acid residues have been deleted, inserted, or replaced with substitute amino acids,
- (3) an FRB domain encoded by a DNA sequence capable of selectively hybridizing to a DNA sequence encoding an FRB of (iv) or (v);

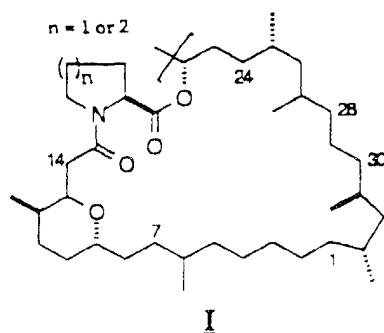
30

and

(b) contacting the cells with an improved rapalog which forms a complex containing itself and at least one molecule of each of the first and second chimeric proteins,

35

where the improved rapalog has an immunosuppressive effect less than 0.01 times that of rapamycin and comprises the substructure of formula I:



bearing one or more optional substituents, optionally unsaturated at one or more carbon-carbon bonds spanning carbons 1 through 8, as a substantially pure stereoisomer or mixture of stereoisomers, or a pharmaceutically acceptable derivative thereof.

2. A method for multimerizing chimeric proteins in cells which comprises:

(a) providing cells which contain:

(i) a first recombinant nucleic acid encoding a first chimeric protein which binds to rapamycin or an analog thereof and which comprises at least one FKBP domain and at least one protein domain heterologous thereto, wherein the FKBP domain comprises a peptide sequence selected from:

- (1) a naturally occurring FKBP
- (2) a variant of a naturally occurring FKBP in which up to 10 amino acid residues have been deleted, inserted, or replaced with substitute amino acids,
- (3) an FKBP encoded by a DNA sequence capable of selectively hybridizing to a DNA sequence encoding an FKBP of (i) or (ii);

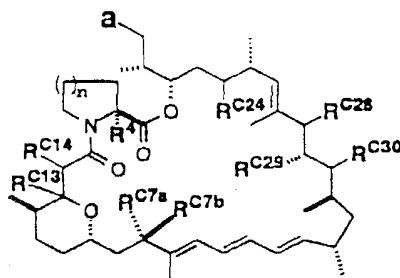
(ii) a second recombinant nucleic acid encoding a second chimeric protein which forms a complex with both (a) rapamycin or a rapamycin analog and (b) the first chimeric protein, and which comprises at least one FRB domain and at least one domain heterologous thereto, wherein the FRB domain comprises a peptide sequence selected from:

- (1) a naturally occurring FRB domain,  
 (2) a variant of a naturally FRB domain in which up to 10 amino acid residues have been deleted, inserted, or replaced with substitute amino acids,  
 (3) an FRB domain encoded by a DNA sequence capable of selectively hybridizing to a DNA sequence encoding an FRB of (iv) or (v);

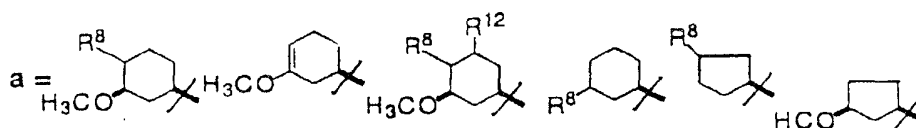
and

- (b) contacting the cells with an improved rapalog which forms a complex containing itself and at least one molecule of each of the first and second chimeric proteins.

where the improved rapalog is of the formula:



wherein



one of R<sup>C7a</sup> and R<sup>C7b</sup> is H and the other is -H, halo, -R<sup>2</sup>, -OR<sup>1</sup>, -SR<sup>1</sup>, -OC(O)R<sup>1</sup>, -OC(O)NHR<sup>1</sup>, -NHR<sup>1</sup>, -NHR<sup>1</sup>R<sup>2</sup>, -NHC(O)R<sup>1</sup>, or -NH-SO<sub>2</sub>-R<sup>1</sup>, where R<sup>2</sup> = aliphatic, heteroaliphatic, aryl, heteroaryl or alkylaryl,

R<sup>C30</sup> is halo, -OR<sup>3</sup> or (=O),

$R^{C24}$  is  $=O$ ,  $=NR^4$ ,  $=NOR^4$  or  $=NNHR^4$ ,  $-NHR^4$  or  $-NHINHR^4$ ,  $-OR^4$ ,  $-OC(O)R^4$ ,

$OC(O)NR^4$ , halo or  $-H$ ,

$R^{C14}$  is  $=O$ ,  $-OR^6$ ,  $-NR^6$ ,  $-H$ ,  $-NC(O)R^6$ ,  $-OC(O)R^6$  or  $-OC(O)NR^6$

5

$R^{30}$  is  $H$ ,  $-R^7$ ,  $-C(O)R^7$  or  $-C(O)NHR^7$  or a cyclic moiety bridging  $C28$  and  $C30$

$R^{C28}$  is halo or  $-OR^3$

10

$R^{C29}$  is  $H$ ,  $OH$  or  $OMe$

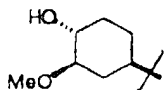
where each substituent is present in either stereochemical orientation unless otherwise indicated, and where  $R^1$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^7$ ,  $R^9$ ,  $R^{10}$  and  $R^{11}$  are independently selected from  $H$ , aliphatic, heteroaliphatic, aryl or heteroaryl;

15

$R^8$  is  $H$ , halo,  $-CN$ ,  $=O$ ,  $-OH$ ,  $-NR^9R^{10}$ ,  $OSO_2CF_3$ ,  $OSO_2F$ ,  $OSO_2R^{4'}$ ,  $OCOR^{4'}$ ,  $OCONR^{4'}R^{5'}$ , or  $OCON(OR^{4'})R^{5'}$ ;

in which one or both of  $R^{C13}$  and  $R^{C28}$  is a halo substituent; both  $R^{C24}$  and  $R^{C30}$  are other than  $=O$ ; one of  $R^{C7a}$  and  $R^{C7b}$  is  $H$  and the other is phenyl, di- or tri-substituted phenyl or a mono- or di-substituted heterocyclic moiety;  $n$  is 1; and/or moiety "a" is other than

20



25

as a substantially pure stereoisomer or mixture of stereoisomers, or a pharmaceutically acceptable derivative thereof.

3. The method of claim 2 wherein  $R^{C13}$  is halo.

30

4. The method of claim 3 wherein  $R^{C13}$  is fluoro.

5. The method of claim 2, 3 or 4 wherein  $R^{C28}$  is halo.

6. The method of claim 3 wherein  $R^{C28}$  is fluoro.
7. The method of any of claims 2 - 6 wherein both  $R^{C24}$  and  $R^{C30}$  are moieties other than  
5 (=O).
8. The method of claim 7 wherein one or both of  $R^{C24}$  and  $R^{C30}$  are -OH, -OR<sup>1</sup> or halo.
9. The method of any of claims 2 - 8 wherein at least one of  $R^{C7a}$  and  $R^{C7b}$  is a moiety  
10 other than -OMe.
10. The method of claim 9 wherein one of  $R^{C7a}$  and  $R^{C7b}$  is H and the other is phenyl, di-  
or tri-substituted phenyl or a mono- or di-substituted heterocyclic moiety.
11. The method of claim 9 wherein one of  $R^{C7a}$  and  $R^{C7b}$  is H and the other is o,p-  
15 dialkoxyphenyl or trialkoxyphenyl.
12. The method of claim 9 wherein one of  $R^{C7a}$  and  $R^{C7b}$  is H and the other is o,p-  
dimethoxyphenyl, o-methoxy-p-ethoxyphenyl, o-ethoxy-p-methoxyphenyl, o,p-  
20 diethoxyphenyl, trimethoxyphenyl or triethoxyphenyl.
13. The method of any of claims 1 - 12 wherein the improved rapalog has an  
immunosuppressive effect less than 0.01 times that of rapamycin.
14. The method of any of claims 1 - 4, 6, 8 or 10 - 13 wherein the chimeric protein encoded  
25 by the first recombinant nucleic acid comprises at least one FKBP domain whose peptide  
sequence contains up to three amino acid replacements relative to a naturally occurring FKBP  
peptide sequence.
15. The method of claim 5 wherein the chimeric protein encoded by the first  
30 recombinant nucleic acid comprises at least one FKBP domain whose peptide sequence  
contains one amino acid replacement relative to a naturally occurring FKBP peptide  
sequence.

16. The method of claim 7 wherein the chimeric protein encoded by the first recombinant nucleic acid comprises at least one FKBP domain whose peptide sequence contains one amino acid replacement relative to a naturally occurring FKBP peptide sequence.
- 5 17. The method of claim 9 wherein the chimeric protein encoded by the first recombinant nucleic acid comprises at least one FKBP domain whose peptide sequence contains one amino acid replacement relative to a naturally occurring FKBP peptide sequence.
- 10 18. The method of claim 14 wherein the chimeric protein encoded by the first recombinant nucleic acid comprises at least one FKBP domain whose peptide sequence contains a replacement amino acid for Phenylalanine-36 of a naturally occurring FKBP peptide sequence.
- 15 19. The method of any of claims 15 - 17 wherein the chimeric protein encoded by the first recombinant nucleic acid comprises at least one FKBP domain whose peptide sequence contains a replacement amino acid for Phenylalanine-36 of a naturally occurring FKBP peptide sequence.
- 20 20. The method of any of claims 1 - 4, 6, 8, 10 - 13 or 15 - 18 wherein the chimeric protein encoded by the second recombinant nucleic acid comprises at least one FRB whose peptide sequence contains up to three amino acid replacements relative to a naturally occurring FRB peptide sequence.
- 25 21. The method of claim 5 wherein the chimeric protein encoded by the second recombinant nucleic acid comprises at least one FRB whose peptide sequence contains one amino acid replacement relative to a naturally occurring FRB peptide sequence.
- 30 22. The method of claim 7 wherein the chimeric protein encoded by the second recombinant nucleic acid comprises at least one FRB whose peptide sequence contains one amino acid replacement relative to a naturally occurring FRB peptide sequence.
- 35 23. The method of claim 9 wherein the chimeric protein encoded by the second recombinant nucleic acid comprises at least one FRB whose peptide sequence contains one amino acid replacement relative to a naturally occurring FRB peptide sequence.

24. The method of claim 14 wherein the chimeric protein encoded by the second recombinant nucleic acid comprises at least one FRB whose peptide sequence contains a replacement amino acid for one or more of Tyr2038, Phe2039, Thr2098, Gln2099, Trp2101 or  
5 Asp2102 in a naturally occurring FRB peptide sequence.
25. The method of any of claims 1 - 24 wherein at least one of the chimeric proteins comprises an action domain which is a DNA-binding domain, transcription activation domain or a cellular signaling domain for triggering growth, proliferation, differentiation or  
10 apoptosis upon dimerization with another protein containing at least one such signaling domain.
27. The method of any of claims 1 - 25 wherein the cells are grown in a culture medium  
15 and the contacting with an improved rapalog is effected by adding the improved rapalog to the culture medium.
28. The method of any of claims 1 - 25 wherein the cells are present in a whole organism and the contacting with an improved rapalog is effected by administering the  
20 improved rapalog to the organism.
29. The method of claim 28 wherein the cells are mammalian and the organism is a mammal.
- 25 30. The method of claim 29 wherein the cells are of primate origin and the organism is a primate.
31. The method of claim 30 wherein the primate is a human.
- 30 32. The method of any of claims 29 - 31 wherein the improved rapalog is administered orally.



Figure 1

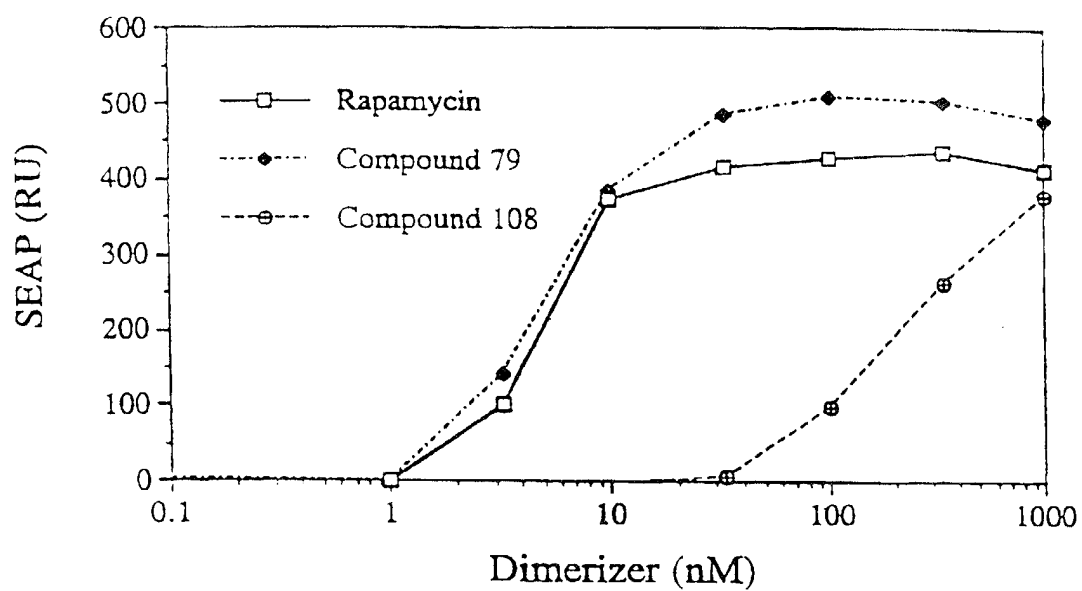
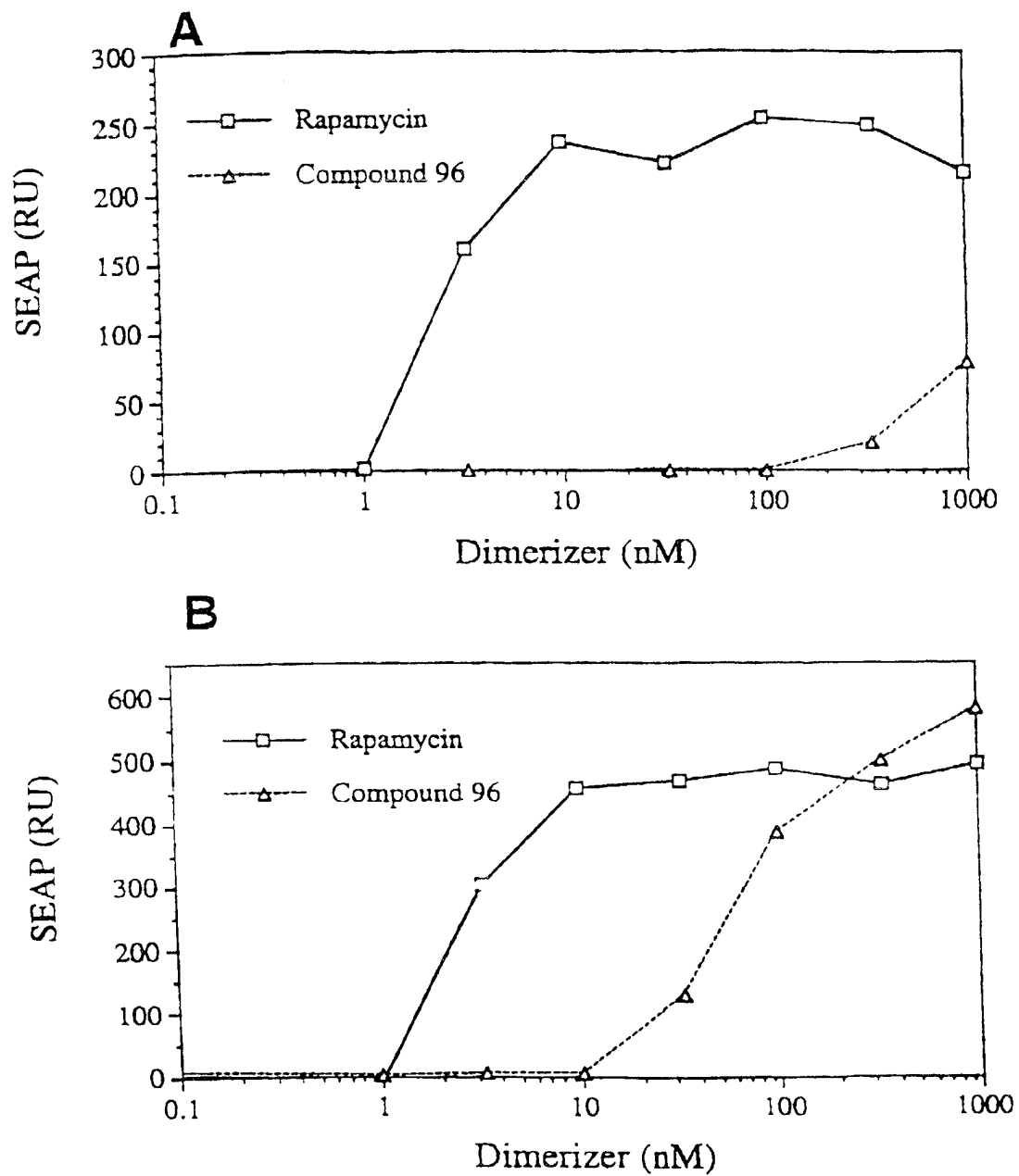
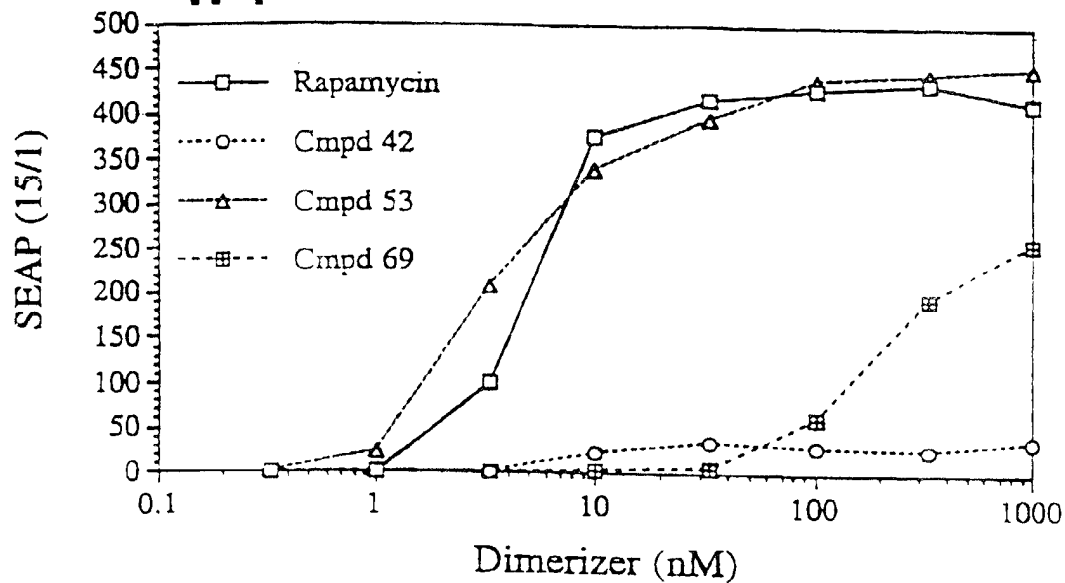
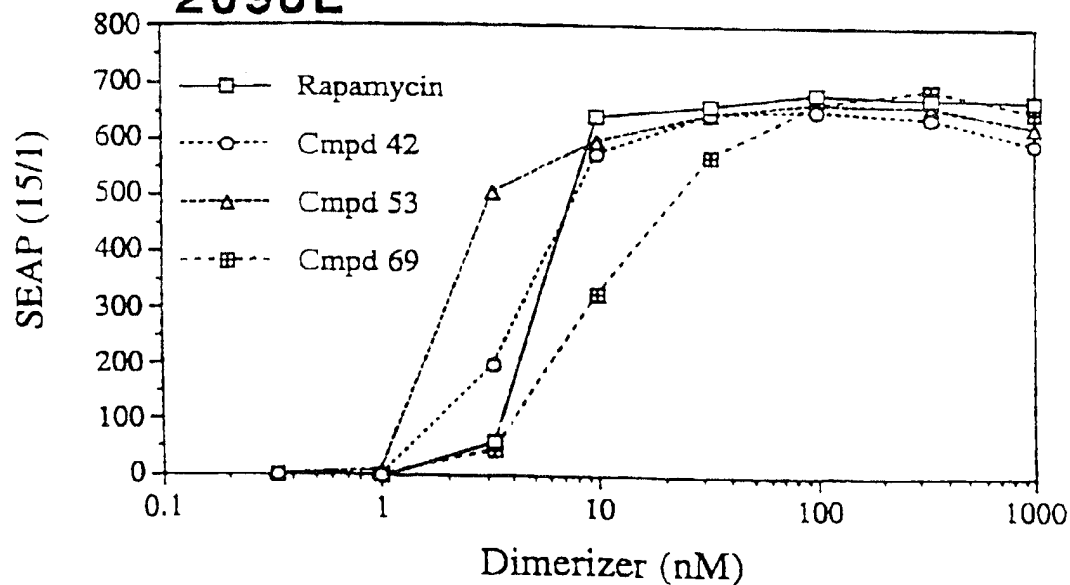


Figure 2



**C****WT****D****2098L**

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